“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Skłodowska-Curie

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Exploring protein production by hydrogen-oxidizing microbiomes

Ir. Silvio Matassa

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Titel van het doctoraat in het Nederlands:

Exploratie van eiwit productie door waterstofgas oxiderende microbiomen

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NOTATION INDEX

ATP	Adenosine triphosphate
CAPEX	Capital expenditure
CAS	Conventional activated sludge
CDW	Cell Dry Weight
CHMP	Heterotrophic production of microbial protein with raw sugar
CHHP	Combined heat, hydrogen and power
COD	Chemical oxygen demand
CR	Continuous reactor
CSTR	Continuously stirred tank reactor
DM	Dry matter
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
GHG	Greenhouse gas
GMO	Genetically modified organism
HOB	Hydrogen oxidizing bacteria
HOMP	Hydrogen-based production of microbial protein
HRT	Hydraulic retention time
LLMP	Land-free production of microbial protein
MAgPIE	Model of Agricultural Production and its Impact on the Environment
MOMP	Methane-based production of microbial protein
MP	Microbial protein
OD	Optical density
OPEX	Operational expenditure
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
RNA	Ribonucleic acid
rpm	Rounds per minute

SBR	Sequencing batch reactor
SCP	Single cell protein
SRT	Sludge retention time
SSPs	Shared socio-economic pathway
TSS	Total suspended solids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

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CHAPTER 1

INTRODUCTION

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CHAPTER 1
INTRODUCTION

Abstract

Microbial biotechnology has a long history of producing feeds and foods. The key feature of today's market economy is that protein production by conventional agriculture based food supply chains is becoming a major issue in terms of global environmental pollution such as diffuse nutrient and greenhouse gas (GHG) emissions, land use and water footprint. Time has come to re-assess the current potentials of producing protein-rich feed or food additives in the form of microalgae, yeasts, fungi and plain bacterial cellular biomass, producible with a lower environmental footprint compared to other plant or animal-based alternatives. A major driver is the need to no longer disintegrate but rather upgrade a variety of low value organic and inorganic side-streams in our current non-cyclic economy. In this context, microbial bioconversions of such valuable matters to nutritive microbial cells and cell components are a powerful asset. The worldwide market of animal protein is of the order of several hundred million tons per year, that of plant protein several billion tons of protein per year; hence the expansion of the production of microbial protein does not pose disruptive challenges towards the production of the former mentioned type of protein. Besides protein as nutritive compounds, also other cellular components such as lipids (Single Cell Oil), polyhydroxybuthyrate (PHB), exopolymeric saccharides (EPS), carotenoids, ectoines, (pro)vitamins and essential amino acids can be of value for the growing domain of novel nutrition. In order for microbial protein as feed or food to become a major and sustainable alternative, addressing the challenges of creating awareness and achieving public and broader regulatory acceptance are real and need to be addressed with care and expedience.

1.1 Introduction

From the times when our ancestors decided to settle, growing crops and domesticating animals became consolidated practices allowing constant feed and food production. As human civilization proceeded, new strategies of securing food supply have continuously been discovered, consolidated and improved. The major driver of such process was the need to provide resilience towards the changing elements of nature, continuously threatening food supply [1].

The current anthropogenic pressure on earth's finite resources and the concomitant dynamics of climate change, generate serious concerns about the resilience of the contemporary agricultural feed/food chains [2]. In view of the still growing world population towards 10 billion in 2050 [3], it has been calculated that the world will need to produce about 70% more food calories than in 2006 [4]. Therefore, there is a need to find reliable alternative solutions, able to strengthen future food security while minimising the impact on the global sustainability.

Microorganisms have always been central in basic food processing techniques, for instance converting fibres into edible food when fermenting dough to produce bread, or milk into cheese, allowing its long-term preservation [5]. They have been often used as direct food source, as it is the case for yeast or microalgae. The latter, together with bacteria, constitute the microbial actors involved in processing food. They can also be used directly as feed or food source [6]. The term "microbe" is used here in the broad connotation of bacteria, fungi, yeast and microalgae.

In the early '60s, when public awareness grew in respect to the impeding global demographic boom, the need to search for alternatives to sustainably feed a growing population corresponded in major efforts to develop alternative feed and food sources [7]. Several attempts were made to develop and bring to practice the production of high-quality protein additives from microorganisms, known as Microbial Protein (MP), or Single Cell Protein (SCP), mainly by using abundant and low cost hydrocarbon substrates such as methanol and methane [7]. The Imperial Chemical Industries Ltd (ICI) were the first to bring to full scale production and commercialization a microbial protein product called Pruteen®, produced from methanol oxidation by means of *Methylophilus methylotrophus* [8]. Besides industrially developed hydrocarbon-based MP, researchers investigated a whole range of other possibilities to produce MP, including the use of natural or artificial light, molecular hydrogen and many different

organic substrates such as by-products from the sugar industry as well as other food processing residues or even food wastes [6]. Despite being well accepted and successful in many feed trials with livestock, the actual and definitive breakthrough of MP in the animal feed market was hampered by the low prices achieved by more conventional protein sources such as soybean and fishmeal in the late ‘70s as well as the fairly underdeveloped state of fermentation technology. Concomitantly, the rising oil prices in the subsequent decades led to the end of the ICI enterprise because of the relatively high costs of MP production and the consequent competitive disadvantage towards other cheaper more “natural” alternatives [9].

In recent years, however, research and development around MP is regaining momentum both in the scientific and industrial domains. The steep increase of the prices of fishmeal (from about $500 per ton in the 1990s to $1500 to $2500 in recent years), together with the environmental pressure of soybean production on land and water use in the tropical areas of the globe justify the re-examination of the microbial alternative [10].

In the present article we align the possibilities offered as well as the challenges to be faced by the use of MP production as a biotechnological tool to help securing nutritive protein supply in the years to come. Threatened by forthcoming population growth, climate change and agricultural unsustainability, mankind must seek, once more, new forms of adaptation to safeguard itself.

1.2 Microbial Protein: feed, food and further

1.2.1 MP as feed

The main driver leading to the renaissance of MP as a source of feed is indubitably the aquaculture sector. Fish farming currently provides about 50% of world’s fish food supply, and it is projected to grow further, becoming a key sector in the supply of high-quality protein for the global population. In this context, scientific research and the industrial applications have found in MP a powerful ally. Aquaculture accounts nowadays for more than 73% of the global fishmeal consumption, with wild fish capture clearly unable to provide enough high-quality feed for such a fast growing sector [11]. Production of MP from natural gas has recently received a great deal of attention, with innovative fermentation processes allowing high volumetric productivities (3-4 kg MP

dry matter (DM) per m^3 reactor volume per hour) by continuous cultures of *Methylococcus capsulatus*, marketed under the name of FeedKind™ [12]. The latter level of productivity has a physical footprint which is a factor 1000, or more, smaller than any conventional vegetable protein production system [13]. Besides achieving feasible industrial scale production and costs competitiveness with fishmeal, the final MP product is comparable to fishmeal in terms of essential amino acid profile and overall nutritive value, also supported by the presence of useful vitamins and other micronutrients [9]. Being tested in numerous feed trials with different fish species, resulting in promising perspectives, full scale production is currently ongoing, with a production of up to 80 000 ton DM/year foreseen in the near future (see Table 1.2).

In addition to aquaculture, the MP product has also been successfully tested in feed trials with terrestrial animals including major livestock like ruminants, pigs and chickens, broadening its potential market applications [9]. In this case though, the relatively low price of soybean meal and the abundant and well established use as main protein additive in livestock production of the latter, still counteract the application of natural gas based MP as replacement of substantial percentages of feed composed by fishmeal.

An alternative route to produce MP consists of recovering valuable nutrients from various side streams of the food industry, for instance feed and food processing water [14]. In this case, the use of heterotrophic microorganisms such as yeast and bacteria allows to convert the organic carbon and the nutrients (N, P) in the waste or processing waters into MP [6]. Microbial protein produced along this line might constitute a valuable and competitive route to produce a substitute for soy protein for animal feed. Indeed, it should be possible to generate such MP at costs which take into account the revenue from the avoidance of the treatment of the mineral nutrients (N, P) present in side (waste) streams. As a matter of fact, dissipation of reactive nitrogen back to atmosphere as dinitrogen gas by means of the conventional nitrification-denitrification pathway comes to a cost of about 2-3 Euro per kg nitrogen-N, whilst capture of phosphorous in the wastewater line costs of the order of 7 Euro per kg P [15]. Note that the market value of proteinaceous nitrogen from vegetable sources is at the current price of some 1.1 – 1.6 Euro per kg dry weight protein (see Table 1.1) corresponding to some 6 Euro per kg proteinaceous N. When this microbial proteinaceous N is converted to high-value animal protein, one can attain an end-value of the same order and even up to 14 Euro per kg dry weight protein, in case of fish.

An example of the implementation of such approach to food processing water is the study published by Lee et al. (2015), where the effluent of a brewery is used as feedstock for the production of SCP-MP. The latter study relates to a technology implemented by Nutrinsic, dealing with a production volume of 5000 ton DM/year from a brewery effluent (see Table 1.2). Also, at present in Belgium a first full scale MP production installation is under construction dealing with the upgrading of potato process waters. It should be in production by 2016 at the level of 5000 ton MP per year (Valpromic NV, personal communication). Yet, for the latter bacterial based MP products, there are so far no clear cut data in terms of their putative market size nor market values. Nevertheless, the sector is attracting growing interests from investors dealing with novel aspects of the cyclic economy [16].

Table 1.1. *Production volumes and price of various animal and vegetable protein sources*

Protein source		Production volume (Mton dm/y)	Farm gate price ($/kg dm)	Average protein content (% dw)	Price per unit protein ($/kg protein dm)	Ref
Animal	Fish	66.7	2.07	15-20	10 - 14	[17]
	Pork	108.5	1.54	20	7.7	[17]
	Chicken	92.7	1.43	31	4.6	[17]
	Beef	62.7	2.70	25	10.8	[17]
Vegetable	Soybean	320.2	0.37	35	1.1	[18, 19]
	Wheat	712.7	0.19	12	1.6	[20, 21]

1.2.2 MP as food

MP is an alternative source of high-quality protein able to replace animal protein like fishmeal in livestock nutrition and aquaculture. Going one step higher in the food chain, MP is meeting the FAO/WHO requirements in terms of essential amino acid scoring pattern for human nutrition (Figure 1.1) and therefore, also humans could benefit greatly from the use of MP directly as food.

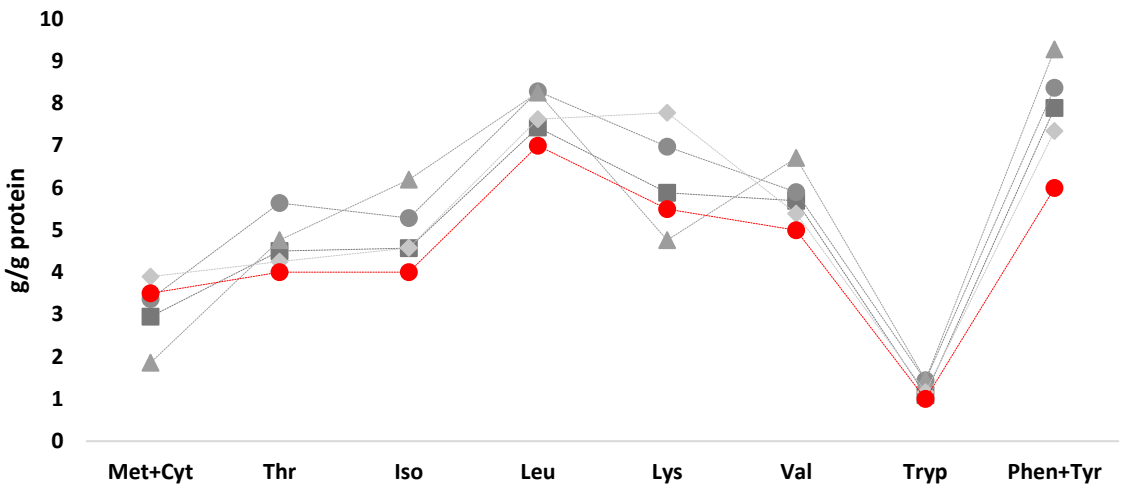

Figure 1.1. *Essential amino acid scoring pattern of microbial protein from bacteria (Pseudomonas/Methylophilus spp.) (■), yeast (Candida spp.) (●), microalgae (Spirulina maxima (▲), compared with the high-quality animal protein from fishmeal (◆) as well as to the FAO/WHO standard (●) for amino acid scoring pattern for human nutrition. Source* [22, 23]

Microalgae are reported to have supported the life of ancient populations living close to the sea for millennia, providing a constant source of protein and vitamins. They are currently used as food and food supplements in food industry [6, 24], with a global production achieving 9000 ton DM/year (see Table 1.2) with a market value estimated about 2.4 billion Euro with a projected yearly growth of 10%.

Yeast, being at the base of food processing since the first bread was baked, or grapes fermented, can also be used as direct food source, as it was the case e.g. with the massive campaign of yeast production and supply, first to the army, then to the whole population during World War II [25]. Currently yeast is a major player in the microbial derived production of products for food as well as for other applications. Baker's yeast and alcohols fermentation are the two main processes employing yeast, with a projected global market value for 2019 of up to 9.2 billion Euro and an annual growth forecast of 7.9% (see Table 1.2).

Fungi are also a suitable alternative and have also made their way as human food. Quorn™ is the most successful example of the so called mycoprotein, which is commercialized and sold in some 15 countries worldwide [26]. Mycoproteins are particularly suited to reproduce the taste and consistency of meat; this explains their success as alternative to conventional animal based products. Currently mycoprotein production supporting Quorn™ products manufacturing amounts to 25 000 ton

DM/year, with a global market value of about 214 million Euro, prospected to grow with 20% annually in the coming years.

Table 1.2. *Overview of current production volumes and market sizes for different microbial protein. Hyphens indicate that values were not available.*

Organisms	Production volume (ton DM/y)	Production costs (Euro/kg DM)	Global market value (Billion Euro)	Yearly growth (% per year)	Advantages	Disadvantages	Remarks	Ref
Yeast	3 000 000	-	9.2	7.9	Easy to harvest; wide range of substrates	Lack of S-containing amino acids	Mostly commercialized as baker's yeast and for ethanol fermentation. Global market value projected to 2019	[6] [27]
Microalgae	9 000	4-25	2.4	10	Natural light as substrate	Low growth rate; High contamination risks; High land footprint; Hard to harvest	Besides feed and food, derivatives are also used in	[6] [28]
Mycoprotein (Quorn®)	25 000	-	0.214	20	Easy to harvest; use of lignocellulosic substrates	Low growth rate; Presence of mycotoxins	Investments for a plant of 22000 tons per year were done in 2015	[6] [29]
Bacteria (Profloc®)	5 000	1-1.1	-	-	High growth rates; use of inorganic substrates	Hard to harvest; Lack of S-containing amino acids; Presence of endo and exotoxins		[6] [30]
Bacteria (FeedKind®)	80 000	-	-	-			Commercial production foreseen on 2016	[6] [31]
Bacteria (Valpromic)	5 000	-	-	-			Personal communication	[6]

1.2.3 Added value applications

Besides being rich in nutritive protein, microorganisms offer the possibility of producing a broad variety of added-value products, suitable for both animal and human nutrition [32]. Table 1.3 summarizes the average amount of protein producible by microalgae, fungi and bacteria, as well as other possible added-value products already investigated or produced from microorganisms.

Certain microalgae and cyanobacteria are primary producers of microbial oil, suitable as substitutes for vegetable oil in food supplements. Particularly, the high concentration of fatty acids can replace fatty acids otherwise derived from rape seed, soy, sunflower oil and palm oil. The purification of omega-3 fatty acid can offer even higher value applications, e.g. for clinical purposes. eicosapentanoic acid (EPA) and decosahexaenoic acid (DHA), normally obtained from fish oil, can be also concentrated

and purified from naturally omega-3 accumulating microalgae. Vitamins such as vitamin B12 and provitamin A are also an important high-value products obtainable from edible microalgae, conferring additional nutritional benefits in livestock production. Carbohydrates, which can be accumulated up to 70% of the cell dry weight by many algal species are also of nutritional value, but the major research effort so far was directed towards the use of microalgae for biofuel, biogas or biohydrogen generation [33, 34].

Fungi, mainly yeast-like fungi are the main agents involved in the saccharification of fibres from corn, as well as fermentation of other organic substrates. While processing corn fiber with yeast like *Aureobasidium*, xylose, arabinose and glucose can be produced at different relative concentrations depending on the pre-treatment of the fibres feedstock. The sugars can then be further fermented in bioethanol, xylitol and pullulan. Xylitol and pullulan find particularly application as food additive for their specific property of flavour-enhancing and binding agents. Besides sugars and sugars-derivatives, yeasts like *Phaffa rhodozyma* (now Xanthophyllomyces rodochrous) can be used to produce valuable carotenoid pigments like astaxanthin, mainly used in aquaculture as feed supplement for salmon [35].

Bacteria are a versatile group of microorganisms able to produce a large array of added-value bio-products. Biopolymers such as polyhydroxyalkanes (PHA) are named to be biological alternatives to petroleum-based based chemicals to produce plastics. Yet, so far large scale applications are to the best of our knowledge, not industrially established. Recently other applications for PHA / PHB such as for example in the medical field are emerging. Of interest for aquaculture is the ongoing research on the prebiotic effects of PHB when used as feed supplement, offering an interesting alternative to antibiotics [36, 37]. Another interesting niche product which can be derived from bacteria are osmo-protectants such as glutamate and ectoine [38]. The latter is a high value cyclic amino acid used in cosmetic formulation, but which has found application also in aquaculture as highly active protectant against oxidative stress. For example, it was observed that hydroxyectoine is able to offer an indirect antioxidative effect by protecting oxidizing groups in the DNA, whereas other cyclic amino acids act directly as antioxidative protectants by scavenging superoxide radicals [38]. Bacteria can also produce relevant amounts of lipids, commonly employed in biofuel production. High quality membrane-derived lipids can be also employed as

human health supplement, being already tested as effective in reducing plasma cholesterol during animal tests [39].

Table 1.3. *Overview of different microorganism for MP and added-value product formation*

Microorganism	Average crude protein content (% CDW)	Nutritional value	Added value by-products (% CDW)	Remarks	Ref.
Microalgae	40-60	Compares favourably to egg, soy and what protein. Cell wall digestibility is an issue	• Microbial oil (50-70%) • Carbohydrates (up to 70%) • Vitamins • …	Triacylglycerides (TAG) can replace partly vegetable oils in food products. Poly unsaturated fatty acids (PUFA) are of interest for health applications.	[33, 40]
Fungi (Filamentous and Yeast)	30-70	Amino acids and digestibility of mycoprotein is similar to egg and milk	• Carbohydrates • Pullulan • Xylitol • Astaxanthin • …	High unsaturated/saturated fatty acids and low fat content makes them highly suitable for human nutrition.	[41]
Bacteria	50-83	Amino acids and digestibility is similar to those of fishmeal	• Internal storage polymers (PHB) • Ectoine • Lipids • Extracellular polysaccharides (EPS) • Growth media and vitamins • …		[39]

1.3 Forthcoming challenges

The extensive use of MP products as partial replacement of conventional protein feed additives such as soybean and fishmeal can offer the opportunity of decreasing part of the environmental pressure that these products exert on land and water use. A recent report of the British Carbon Trust evaluated the environmental impact of FeedKind™ protein, a bacterial MP feed additive produced from natural gas (see section 2.1). The report evaluated two FeedKind™ commercial products in terms greenhouse gas, land and water use, comparing them with soybean and fishmeal [42]. In terms of fresh water consumption, the report shows an average value of about 29 m^3/ton MP produced. A more detailed analysis shows that this 29 m^3 is for about 80% determined by the

vegetable oil used as binding agent to produce a MP pelletized product. If the latter major contribution is excluded by producing a simple straightforward protein powder, the actual fresh water requirement comes down to the order of 1 m^3/ton MP. From Figure 1.2 it can be derived that this value about water foot print is about 20 and 140 times lower than fishmeal and soybean meal, respectively.

The same trend is observable for the required land. The value of 52 m^2/ton MP is in fact due to vegetable oil for the production of the pelletized form, whereas no arable land is required in case of the powdered MP. Compared to the 6 655 m^2 land/ton protein required for the production of soybean meal concentrate, the value of quasi zero land foot print reveals how the land footprint of MP production is a major benefit in respect to conventional agricultural based protein production. Fishmeal of course requires minimal amount of land for its processing, yet the dramatic impact of wild fish capture on ocean ecosystems is well known and documented [43].

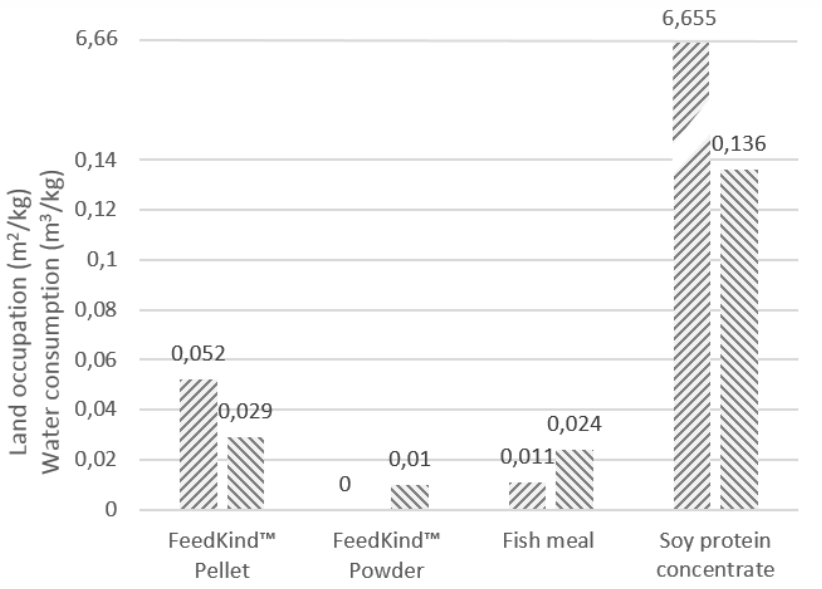

***Figure 1.2**. Land (▨) and fresh water (▨) requirements of MP compared with fishmeal and soy protein concentrate. The values are normalized to the protein content of each product. Source: Cumberlege et al.* [42].

Finally, the above mentioned report also analyses the carbon footprint of FeedKind™. The value of 5.8 ton CO_{2eq}/ton MP is mainly due to the natural gas necessary for the metabolism of the bacteria involved in the biological fermentation process. This value would be as low as 1.7 ton CO_{2eq}/ton MP in case biogas and renewable energy is used in place of fossil fuels to power the reactor-based production and downstream processing of the final MP product. For fishmeal and soybean meal concentrate, the

report indicates values of 2.6 and 0.8 ton CO_{2eq}/ton protein, respectively. Nevertheless, if the spared agricultural land in case of MP production would be accounted for its recovered carbon capture potential, the overall benefit in avoided carbon emissions from MP production could possibly be much higher.

In this context, an interesting alternative to natural gas-based MP is represented by autotrophic microorganisms such as microalgae or hydrogen-oxidizing bacteria. If microalgae offer the great advantage of being able to use sun light to fix carbon dioxide into biomass, the main drawbacks of such process are the high land footprint required together with the technical challenges of downstream processing of poorly concentrated algal biomass [44]. On the other hand, the hydrogen gas needed to fix carbon dioxide into bacterial biomass by means of hydrogen oxidizing bacteria is a more expensive resource, but the land footprint and the biomass concentrations achievable with modern fermentation technologies outscore those of the algal platform. In case the production of the latter is connected to hydrogen generated by means of renewable energies (solar, wind, etc.), this allows an elegant platform for MP production and concomitant carbon dioxide capture [45].

To the scientific community, it is evident that a dedicated industrial production of MP can represent a key biotechnological tool to curb down the environmental impact of the current feed and food chain assuring the necessary amounts of nutritive protein for mankind. Clearly, significant efforts are warranted to bring this to practice at relevant scales. A key feature is to deal with the aspects of public awareness. At present, the mere economic market rules justify the application of MP in feed for livestock only in some niche applications such as aquaculture. Yet, if the externalised environmental costs of the current feed/food production system would be taken into account and made clear to the broader public (including decision makers in political institutions), the MP route would result in a more rational alternative, able to offer immediate advantages in terms of water and land use, with direct consequences on increased carbon capture potential of ecosystems restored by better agricultural land use [46]. An important aspect, in this sense, relates to nutrients flows, and principally the excessive input in our biosphere of reactive nitrogen species (NH_4^+, NO_2^-, NO_3^-) produced by fixing atmospheric N_2 gas by means of the Haber-Bosch process. Compared to the proposed sustainable boundary of 35 Mton N_2 fixed per year, the current 121 Mton actually converted into reactive nitrogen surpass the sustainability boundary of almost 3.5 times [47]. Moreover, if the economic benefit in agricultural

production ranges between 20-80 billion Euro per year, the annual costs (including damages to both environment and human health) of N pollution by agriculture have been estimated in the range of 35-230 billion Euro per year [48]. It has been recently demonstrated how the high nitrogen inefficiency of the soil-plant system could be mitigated by MP production [13], decreasing sensibly the impact of eutrophication, nitrous oxide emissions and ecosystems disturbance due to unbalanced anthropogenic nitrogen inputs.

Besides the awareness of the overall environmental benefit of MP production for feed and food, the development of higher-value by-products will allow boosting and bolstering the MP biotech platform. Thus a more powerful penetration into the market of microbial based product as replacement of chemically derived ones, as discussed above, will be possible. This will play in favour of establishing a public mind set more open and prone to acceptance towards microbial derived products.

Obviously, barriers must be overcome in order to allow a widespread adoption of the MP biotechnology. Besides the official legal recognition of some MP products as feed and food [9], further openings are warranted in terms of used carbon and nutrient sources recovery and their up-cycling into edible MP products as part of the cyclic economy. This will impact drastically on how efficiently our current society makes use of its precious primary resources.

1.4 Aims and outline of this work

The aim of the research presented in this thesis is to investigate the production of microbial protein by means of a hydrogen oxidizing bacteria microbiome enriched from a generic environmental sample. The effect of different engineered reactor systems on the biotech performances as well as on the microbial community composition of the microbiome have also been studied. The potentialities of such biotech process in terms of resource recovery and upcycle (nitrogen but also carbon dioxide) are discussed throughout the whole thesis and compared with other microbial platforms, both at WWTP scale as well as within a broader global agricultural integrated model.

Chapter 2 introduces hydrogen oxidizing bacteria (HOB) starting from their most basic physiological aspects. Once their characteristics have been outlined, the concept of

nitrogen recovery into microbial protein by means of HOB is discussed and first estimations are made in terms of process efficiency and potentialities.

In **chapter 3** the scientific approach foreseeing the use of microbiomes instead of axenic cultures followed in this thesis is introduced. First the methodology used to enrich the HOB from an environmental sample is presented and then the results of the enrichment process are discussed. Some preliminary physiological and kinetic parameters of the HOB microbiome are analyzed and discussed, constituting the basis for upscaling the experiment from lab to bench-scale.

Chapter 4 presents and discusses the bench scale experiments conducted on the enriched HOB microbiome. The main biotech parameters such as biomass yield, volumetric productivities and gas utilization efficiency are compared between two specific engineered reactor systems: the sequencing batch reactor (SBR) and the continuous reactor (CR). The effect on the microbial community composition of the latter systems is analyzed and innovative outcomes in terms of novel fully aerobic HOB species are discussed. Moreover, the final quality of the produced microbial protein is evaluated in relation to the essential amino acid composition analysis.

In **chapter 5** the upcycling of nitrogen recovered from used water for the production of microbial protein by means of HOB and other microbial platforms is discussed. More specifically, the nitrogen use efficiency of the current feed and food chain is compared with the efficiency offered by shortcutting the conventional agro-food line by means of direct nitrogen recovery and upcycle by means of microbial protein.

Based on what is presented in chapter 5, **chapter 6** presents a case study where the HOB-based microbial protein platform is implemented in the upcycling of nitrogen recovered from anaerobic digestion. In this case, the “water factory” maximizing C and N recovery is sketched and implemented with renewable hydrogen generation platforms, used to capture upcycle CO_2 and N under the form of microbial protein.

Similarly to chapter 5, **chapter 7** compares different microbial platforms for the production of microbial protein. In this case though, the production of microbial protein is analyzed starting from industrially synthesized nitrogen, i.e. Haber-Bosch produced

nitrogen, allowing a direct comparison of the microbial protein platform with the conventional agro-feed-food based system. A thorough economic analysis is used as input for the global agricultural model MAgPIE, which allows to confront the different platforms in terms of nitrogen use efficiency, greenhouse gas emissions and land use efficiency on a global scale.

In Chapter 8 a final discussion addresses future challenges and perspectives of the work presented in this thesis.

Chapter

2

Resource recovery from used water: the manufacturing abilities of hydrogen-oxidizing bacteria

Chapter redrafted after:

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CHAPTER 2

RESOURCE RECOVERY FROM USED WATER: THE MANUFACTURING ABILITIES OF HYDROGEN-OXIDIZING BACTERIA

Abstract

Resources in used water are at present mainly destroyed rather than reused. Recovered nutrients can serve as raw material for the sustainable production of high value bio-products. The concept of using hydrogen and oxygen, produced by green or off-peak energy by electrolysis, as well as the unique capability of autotrophic hydrogen oxidizing bacteria to upgrade nitrogen and minerals into valuable microbial biomass, is proposed. This process can become a major line in the sustainable “water factory” of the future.

2.1 Introduction

The present approach to wastewater treatment is burdened by the heritage of the sanitary engineering: disintegrate the residual materials, make them disappear. In the conventional activated sludge (CAS) system, the wastewater is treated by means of an energy-demanding dissipative set of processes, aiming at the total decomposition of all organic molecules prior to return of the cleaned effluent to the ecosystem [49]. Currently, wastewater is regarded as an assembly of resources to be recovered such as energy, nutrients (C, N, P etc.) and water itself. Each of them can be used as building blocks of all forms of life. The need to fully or partially revise the decomposing and dissipative aspects of the CAS system is currently addressed by scientific research, and this effort is nowadays attracting increasing attention [50–53]. The decrease of the environmental footprint, particularly by limiting greenhouse gas (GHG) emissions, and by focusing on the recovery of valuable resources, are urgent and mandatory issues in wastewater treatment systems [54, 55]. When considering future perspectives towards innovative bio-treatments of anthropogenic waste streams, environmental biotechnology offers a virtually infinite set of bioprocess combination, which can theoretically lead to achieve remarkable results in terms of process innovation and efficiency. The latter and the developments in the field of renewable energies and process engineering, provide plenty of opportunities for serendipities [52]. In this framework, hydrogen-oxidizing bacteria can be considered as one of the most powerful microbial actuators of the transition towards integrated bio-refineries. They are ubiquitous bacteria with the ability to consume molecular hydrogen in their energy yielding process: it gives this group of microorganisms several nutritional advantages such as the ability to grow in an exclusively inorganic medium, converting rapidly CO_2 and reduced nitrogen into new cellular material [56, 57]. Innovative approaches employing this kind of bacteria might be suitable for the upgrade of nutrients recovered from anaerobic digestate and reject waters in wastewater treatment plants (WWTP), as well as for carbon dioxide capture and upgrading in the process of converting biogas to biomethane. Implementing a circular approach, the basic components to be removed from liquid or gaseous streams would be not anymore “neutralized” but recovered and upgraded into new valuable microbial biomass rich in proteins, biopolymers or microbial oil. This mini-review aims to outline some of the main features and perspectives of hydrogen-oxidizing bacteria. It should

be emphasized that these bacteria have been explored extensively almost half a century ago as potential working horses for microbial technology. The concept is that at this age of bio-economy and with the global feed and food market in strong need of alternative protein sources, they can potentially find effective niches for useful application in the context of resource recovery from used water.

2.2 Hydrogen-oxidizing bacteria

Hydrogen-oxidizing bacteria or Knallgas bacteria (named after the gaseous mixture of H_2 and O_2 they consume) are aerobic, facultative autotrophic bacteria which share the ability to fix carbon dioxide into new cellular material by the ribulose biphosphate or reverse tricarboxilic cycle, using hydrogen and oxygen, respectively, as electron donor and electron acceptor in the energy yielding process [58]. Indeed the change in Gibbs free energy and the resulting ATP formation at pH 7 (with a negative change in free energy) is substantial (Eqs. (1), (2) and (3)) [59],

$$H_2\,(g) + ½\,O_2\,(g) \rightarrow H_2O\,(L) \qquad \Delta G^0 = -237.1\ kJ/mole \qquad (1)$$

$$ADP + Pi \rightarrow ATP \qquad \Delta G^0 = +30.5\ kJ/mole \qquad (2)$$

$$H_2\,(g) + ½\,O_2\,(g) + 7\,(ADP + Pi) \rightarrow H_2O + 7ATP \qquad \Delta G^0 = -23.6\ kJ/mole \qquad (3)$$

Besides possessing the key enzymes that allow them to grow with H_2 + CO_2 as the sole energy and carbon sources, these aerobic bacteria can support their growth also by oxidizing organic substrates such as sugars, organic acids and amino acids [60], therefore possessing mixotrophic metabolic capabilities. In their study on hydrogen metabolism in aerobic hydrogen-oxidizing bacteria, Schink and Shlegel [60] identified them as a heterogeneous group of taxa such as *Alcaligenes, Pseudomonas, Paracoccus, Aquaspirilium, Flavobacterium, Corynebacterium* (Gram-negative genera) as well as *Nocardia, Mycobacterium and Bacillus* (Gram-positive genera). In general, they are all naturally occurring microorganisms, inhabiting niche environments where oxygen concentrations fluctuate around hypoxic conditions (oxic-anoxic threshold). In this way, they take advantage of the hydrogen released by anaerobic microorganisms without being affected by high O_2 concentrations.

2.2.1 Bio-products from hydrogen-oxidizing bacteria

Hydrogen-oxidizing bacteria are a special group of bacteria which attracted the attention of researchers already during 1970s, as potential producers of single cell protein (SCP) [56], biomass for fermentation industry and polyhydroxybutyrate (PHB) [61] (Figure 2.1).

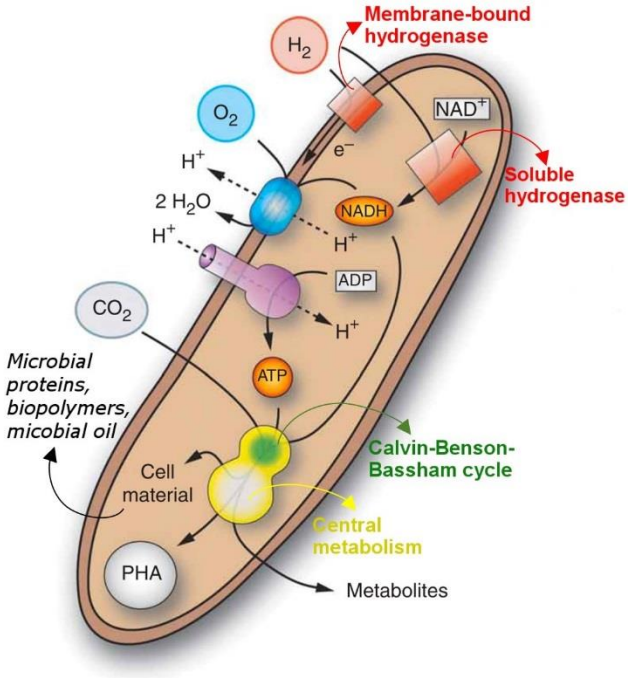

Figure 2.1. *Schematic representation of lithoautotrophic metabolism and bio-product formation in Cupravidus necator. Adapted from Pohlmann et al.* [62].

Autotrophic cultivation of hydrogen-oxidizing microorganisms represents the core of many studies since this peculiar group of bacteria was discovered. The most representative and well-studied hydrogen-oxidizing bacterium is *Cupriavidus necator*, which name underwent several changes along the years: *Hydrogenomonas eutrophus, Alcaligenes eutropha, Wautersia eutropha* and *Ralstonia eutropha* [58]. For reasons of clarity, we further on use most often the most recent name, i.e. *Cupriavidus necator*. The attention paid to this strain is due to its extremely flexible versatile metabolism, i.e. the capability of easily shifting between heterotrophic and autotrophic growth modes, using organic compounds or molecular H_2 as energy sources, both alternatively or concomitantly [62]. The research on bioprocesses related to this microorganism generated an interesting amount of information concerning its stoichiometry and kinetic parameters, which can be regarded as reference points for this group of bacteria.

2.2.2 Stoichiometry of Hydrogen-oxidizing bacteria

The stoichiometry for autotrophic cell growth of *Cupriavidus necator* as indicated by Ishizaki and Tanaka [63] is the following:

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.70\ H_2O \qquad (4)$$

The molar ratio of gaseous substrate consumption ($H_2/O_2/CO_2$) here reported, however, can change when other strains are considered, and depends on the growth conditions and the growth rate [60]. The most common ratios of the gaseous substrate composition reported so far in different studies about hydrogen-oxidizing bacteria are of the order of $H_2/O_2/CO_2$ = 7:1:1 (v/v) [64] or 7:2:1 (v/v) [65]. In this sense, an important metabolic parameter is represented by the H_2 uptake over CO_2 uptake ratio, which is regulated by the balance between the expenditure for catabolic energy and the level at which electrons enter the respiratory chain. H_2/CO_2 values ranging between 4 and 10 were reported as suitable for the growth of these microorganisms [60]. Many studies have indicated the crucial role that the oxygen concentration plays in the metabolism of aerobic hydrogen-oxidizing bacteria. It is necessary as final electron acceptor, but inactivates the hydrogenase enzymes if present above certain limits [66]. Oxygen inhibition of growth was indicated as strain dependent in earlier studies on *Cupriavidus necator* [61], with growth inhibition occurring already at O_2 concentrations of 4% (*v/v*), whereas recently the highly CO-tolerant *Ideonella sp. O-1* was found as capable of growing in presence of O_2 levels greater than 30% (*v/v*) [64].

2.2.3 PHB: from bio-polymers to prebiotic

Amongst the interesting metabolic features of hydrogen-oxidizing bacteria, increasing attention has been paid to the accumulation of biopolymers, particularly by employing axenic cultures of *Cupriavidus necator*. Since earlier studies indicated how far developed this trait was in *Cupriavidus necator*, especially under oxygen limiting conditions [61], the autotrophic cultivation of this microorganisms for PHB production was studied by different groups of researchers [65]. This process was regarded as a possible way of binding CO_2, coupling it to the production of biodegradable and renewable biopolymers [65, 67]. Recently, some studies highlighted also the possibility of using CO-tolerant hydrogen bacteria for PHB production on exhausted industrial emissions rich in H_2, CO_2 and CO [64, 68]. Besides the widespread research on PHB

as raw material for bio-plastic production, these bio-polymers have been recently shown to be able to act as microbial control agents when used in the diet of different aquaculture species [69].Once assimilated in the gut, the biopolymer starts being decomposed into its monomer: butyric acid, thus acting as a slow release carbon source which bolsters the gut microbiota, and resulting in an overall positive prebiotic effect. Therefore, PHB have also the potential to be used as anti-infective agents for aquaculture[36, 37, 70], broadening the possible applications of this microbial byproduct.

2.2.4 Stoichiometry of PHB formation in Hydrogen-oxidizing bacteria

Polyhydroxybutyrate (PHB), is a biopolymer used as energy storage by bacteria. They accumulate excess of carbon in form of biopolymers when low concentrations of other compound such as oxygen or nutrients limit their growth [58]. The stoichiometry of PHB accumulation in *Cupriavidus necator* can be expressed as follows [67]:

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \qquad (5)$$

Hence, 1.30 kg of PHB could be theoretically harvested per kg of H_2 metabolized, which in energy terms corresponds to 0.16 kg PHB/kg H_2-COD. The remarkably high weight of PHB per kg H_2 is explained by to the fact hydrogen is the lightest existing molecule. On a COD basis, the PHB yield by hydrogen-oxidizing bacteria is comparable to other bacteria accumulating PHB from C_1 compounds (0.14 kg/kg methane-COD), whereas it is less favorable if carbohydrates (0.45 kg/kg glucose-COD), C_2 compounds (0.45 kg/kg acetic acid-COD) and C_4 compounds (0.64 kg/kg butyric acid-COD) are used as energy source [71]. This stoichiometry is assumed as representative also of other hydrogen-oxidizing bacteria, able to accumulate PHB under stress conditions.

2.3 Tackling the sustainability of feed production: microbial proteins from H_2, CO_2, NH_3 and O_2

The fixation of carbon dioxide into new cell material, generating new biomass and bypassing the photosynthetic pathway sun-plant-biomass, was the very first focus of early studies on hydrogen-oxidizing bacteria. The capability of these bacteria to rapidly

grow in generic and inexpensive inorganic media (m_{max} = 0.42 h^{-1}) [63], achieving considerably high yields in terms of volumetric production rates (5.23 g CDW/L·h) [67], was investigated in several studies on the production single cell protein to be used as animal feed [56]. Lepidi et al. [72] made a first attempt to compare the energy efficiency of biomass production from hydrogen-oxidizing bacteria with the photosynthetic efficiency of the fastest growing plants. They proposed a process scheme where renewable energy was used for electrolysis of water, producing H_2 and O_2 to be used as gaseous substrates (together with CO_2) for biomass production in reactor systems. The estimated solar energy recovery of 2% as caloric power of microbial biomass was already higher than the photosynthetic efficiency of 0.5% of the fastest growing crop [73]. Notably, the authors concluded that, based on their data several hundred tons of microbial biomass dry matter per ha per year could be produced in well-designed reactor systems [72]. At present, the highest production levels of C_4 plants like e.g. maize are in the range of 10-20 tons dry matter per ha per year [74, 75]; this is a fraction relative to potential that H_2-oxidizing bacteria may have per unit footprint when efficiently grown in reactor systems. Recently, a similar study employing an axenic culture of *Cupriavidus necator* in a closed reactor system estimated a solar energy recovery around 5% [59], and the overall efficiency of the solar hydrogen coupled to the microbial culture system was evaluated to be up to 10 times higher that of the conventional crop plants or microalgae. Besides being a sustainable and efficient alternative to photosynthetic biomass production, hydrogen-oxidizing bacteria can be regarded as a potential source of microbial protein, i.e. single cell protein (SCP) [6]. The suitability of hydrogen-oxidizing bacteria as SCP producers was recently investigated in a study on the characteristics of the proteins synthetized by these bacteria [76]. The biological value of proteins synthetized by three hydrogen-oxidizing bacteria was assessed: *Alcaligenes eutrophus Z1*, *Ralstonia eutropha B5786* and the CO-resistant strain of carboxydobacterium *Seliberia carboxydohydrogena Z1062*. This study showed that the high content of protein synthetized by these bacteria possesses also has a complete profile of valuable amino acids. Indeed, the amino acids profile was similar to that of yeast, microalgae and casein, but the final content of protein of 70 % (on a dry weight basis) for H_2-oxidizing bacteria was much higher than the respective values i.e. 50 and 15 % measurable in yeast and wheat grain. Further, the total essential amino acids content of hydrogenotrophs was more than 10% higher than in grain and close to the content of casein. Moreover, the assimilation in the

gastro-intestinal tract (simulated by availability for proteolytic enzymes *in vitro*) of such microbial proteins is about a factor 1.4 higher than that of wheat proteins and almost comparable to that of casein, which amounts to 44 % for pepsin (after 3 hours) and 55 % for trypsin (after 6 hours) [76]. In view of the increasing scarcity of food proteins (land use limitations, water scarcity, climate change, increased demand [77–80]) and the long-term stability of augmented prices of feed and food on the world market, it stems to reason that at present a renewed attention can be seen in the direction of developing microbiological technologies for protein synthesis on different gaseous and liquid substrates, particularly if the latter can be generated in a sustainable way [81].

2.3.1 New approaches to resource recovery: the H_2-based biorefinery

Table 2.1. *Hydrogen-oxidizing bacteria yields and estimated cost of microbial by-product (downstream processing costs are excluded)*

Process/product	Stoichiometric yield (kg product/kg H_2-COD)	Higher yield observed (kg product/kg H_2-COD)	Final cost [a] (€/kg product)	Market cost	
				Reference product	Cost (€/kg)
Microbial biomass rich in protein	0.28 [63]	0.30 [61, 63]	0.82	Soymeal	0.41 [82]
PHB	0.16 [67]	0.12 [68]	1.44	PHB	1.47 [83] [b]
NH_4^+-N removal and assimilation	0.03 [63]	-	7.67 [c]	N-removal	1.0 - 6.0 (Table 2.3)

Note: *Other currencies were converted accordingly to the exchange rates of ECB* [84]
[a] *Calculated on the stoichiometric yield and the chemicals concerned (opex and capex not included)*
[b] *Possible price of PHB production*
[c] *Cost is intended per Kg of nitrogen removed by microbial assimilation*

The production of hydrogen from renewable energy sources is gradually replacing the generation from fossil fuels driven systems, and the technical advances in the energy sector are expected to lower the prices of green hydrogen production in the near future [85]. For instance, electrical energy efficiencies up to 73% are already achieved by commercial and industrial grade electrolysers, and researches on new materials and electrolysers configurations showed possible efficiencies as high as 96% [86]. Hydrogen has always been regarded as a sustainable alternative to fossil fuels or as

a mean of electrical energy storage [87]. Nevertheless, it can be also seen as a primary energy source in case of hydrogen-oxidizing bacteria. Hydrogen production costs from renewable energy sources vary accordingly to the considered scenario. Cost analyses available in literature suggest values of around 1.7 Euro/kg H_2 for natural gas reforming (which can be also applied to biogas) [85], 1.2 Euro/kg H_2 for biomass gasification [85] and 1.8 Euro/kg H_2 for hydrogen generation from wind energy [88]. The latter was taken as reference to estimate the energetic costs related to the use of hydrogen-oxidizing bacteria, with a final reference value of 0.23 Euro/kg H_2-COD. The microbial performances of hydrogen-oxidizing microorganisms are resumed in the yields reported in Table 2.1, which calculations were based on stoichiometric data reported in literature about *Cupriavidus necator*. The yields were calculated on an equivalent H_2-Chemical Oxygen Demand (H_2-COD) basis. A comparison between the energetic costs related to hydrogen-oxidizing bacteria and the prices of similar products/processes already marketed is also reported in Table 2.1. As indicated, the production costs of microbial biomass rich in proteins are around two-fold the marketable price of soymeal. However, the average protein content of the latter product is of the order of 40% [89], whereas hydrogen-oxidizing bacteria were reported as capable to accumulate as much as 75% of protein on a dry weight basis [76]. These first considerations allow estimating a final raw protein cost comparable to the market price of soymeal used as reference protein feedstuff. Clearly, other opex and capex costs should not be neglected, but the latter are primarily related to the scale dimensions, and should certainly not triple the costs based on those of the primary ingredients i.e. hydrogen and oxygen. More realistic estimations of the final protein cost for raw materials needed in single cell protein production [90] indicates that an amount of 62% of total production costs is related to issues of utilities, labor and supervision, fixed charges, maintenance, etc. In this case, the marketable price of the single cell protein would be around 1.75 Euro/kg of protein, which is a factor 1.7 above the actual price of soymeal. Nevertheless, this estimated marketable price is still lower than e.g. the total production cost of 2.10 Euro/kg dry cell of yeast grown on molasses[91]. When the production of biopolymers as PHB is taken into consideration, the energetic production cost is even lower than what is reported in literature for possible microbial PHB production on other substrates such as glucose and methanol [58]. Here, the calculated cost of PHB production from hydrogen does not consider the treatments costs for the extraction and the separation of the biopolymer from the

microbial biomass. This latter consideration is fully justified when PHB are regarded as prebiotic feed additive, able to confer added nutritional value to the produced microbial cells [36, 37, 69].

2.3.2 The methane and the hydrogen platform: a comparison

The same range of bio-products (SCP, PHB) can be also obtained by exploiting another type of autotrophic bacteria: methane-oxidizing bacteria. These well-studied microorganisms have in the past been implemented in full scale SCP production systems [92], and tested as protein-rich feed additive for cattle and fish [93, 94]. In the bio-refinery context outlined in our work, these bacteria might be easily applied by making use of the large amounts of biogas produced at sewage and manure treatment plants. Compared to hydrogen-oxidizing bacteria, methane oxidizing bacteria generally possess a stricter metabolism (i.e. obligate methanotrophy), with lower biomass yields, and similar PHB yields (see Table 2.2). In addition to the lower biomass yield, they also have lower growth rates and lower protein levels (see Table 2.2). When compared with hydrogen-oxidizing bacteria, they indeed can be set to work directly on renewable resources such as methane produced from biomass either biological or by gasification. Their downsides are i) lower biomass yield which decreases the maximum volumetric loading rate with a factor 1.5 in a cell-retention configuration or with a factor 10 in flow-through configuration (see Table 2.2), leading to an increased footprint, and ii) the purity of the produced feed, which is not guaranteed against residual amounts of alkanes [95]. Overall, the application of one type of autotrophic bacteria does not exclude the concomitant use of the other. In view of a hydrogen driven economy, the faster and more efficiently growing H_2-oxidizing bacteria are of value in the line of new developments in resource recovery from used water, whereas the already established methane-oxidizing bacteria can represent a technology for upgrading low value methane to microbial biomass used as source of bio-products [96].

Table 2.2. *Comparison between methane-oxidizing bacteria and hydrogen-oxidizing bacteria used in SCP and PHB production*

	Methane-oxidizing bacteria	Hydrogen-oxidizing bacteria	Ratio (Hydrogenotrofic ratio/Methanotrofic)
Growth rate (h^{-1})	0.043 [97]	0.420 [63]	9.8
Cell yield (g CDW/g COD)	0.19 [96]	0.28 [63]	1.5
PHB yield (g PHB/g COD)	0.14 [58]	0.16 [67]	1.1
Protein content (% Protein on CDW)	60% [97]	75% [76]	1.3

2.3.3 Re-thinking nutrients dissipation: moving towards nitrogen assimilation and upgrade

The conventional nitrification-denitrification process for nitrogen removal from used water is nowadays challenged by new emerging technologies. Advances in research and in biotechnological applications opened a complete new set of processes for nitrogen removal, able to decrease drastically the demand of consumables (chemicals or carbon sources) and energy supply. In the conventional nitrogen removal process, the reduced ammonium nitrogen is first oxidized to nitrate and then reduced to nitrogen gas in the two-step process of nitrification-denitrification. Since anammox (anaerobic ammonium oxidizing) bacteria were discovered, innovative processes able to short circuit the conventional nitrification-denitrification were developed, tested and implemented up to real-scale applications [98]. Processes based on anammox bacteria are indeed taking over the conventional bioprocess of nitrogen removal, particularly when dealing with high loaded nitrogen water such as sludge reject water [99], landfill leachate and industrial or agricultural effluents [100]. For instance, when sludge reject water is recirculated back in the main treatment line, it can account up to 25 percent of the total influent nutrient in the WWTP [101]. Therefore, the cost-effective treatment of this highly concentrated side streams is an issue of increasing concern.

Table 2.3. *Total cost including capex and opex for processes for nitrogen removal and nitrogen recovery*

N-removal technique	Type of process	N-recovery	Cost per Kg N (Euro)	Reference
Nitrification-denitrification	Biological	No	2.3-4.5	[99]
Anammox	Biological	No	1.0	[100]
Air-stripping	Physical-chemical	Yes: $(NH_4)_2SO_4$	6.0	[102]
MAP	Physical-chemical	Yes: $MgNH_4PO_4 \cdot 6H_2O$	6.0	[102]

Table 2.3 reports a comparison between the costs for nitrogen removal and recovery using different processes. The biological processes reported include the conventional nitrification-denitrification and the innovative systems based on anammox bacteria. The latter are designed in order to obtain maximum nitrogen dissipation from the effluent (by converting dissolved NH_4^+ to N_2 gas). The lower integrated cost per unit of nitrogen removed (up to 5 times less expensive, see Table 2.3) makes anammox-based approaches rather than the thus far implemented (i.e. coupled nitrification and denitrification) the route to go for the treatment of nitrogen-rich side streams such as reject water from sludge treatment plants [103]. Nevertheless, even the latter processes are still relying on the assumption that nitrogen has to be removed rather than recovered. Dealing with resource recovery from used water, nutrients are more and more regarded as potential new building blocks. The ammonia can be upgraded to fertilizer rather than be destroyed for fear that it will cause eutrophication [104, 105]. The other two processes reported in Table 2.3 are physical-chemical systems aiming to both nitrogen removal and recovery. The removal of reduced dissolved nitrogen (i.e. ammonium ions NH_4^+) by means of stripping is based on the increase of pH and temperature of the effluent prior to removal of ammonia gas with air [106]. The subsequent reaction of the stripped ammonia with acids allows to recover the nitrogen as e.g. ammonium sulfate $(NH_4)_2SO_4$, which can be used as soil fertilizer [107]. Removal of reduced dissolved nitrogen can also be performed together with soluble phosphorus in the so-called MAP (Magnesium ammonium phosphate - struvite) process. When dissolved ammonium (NH_4^+) and phosphate (PO_4^{3-}) ions are present together with magnesium ions (Mg^{2+}) in the molecular ratio of 1:1:1, the precipitation

of a crystalline solid allows recovery in form of struvite: $MgNH_4PO_4 \cdot 6H_2O$ [105]. Amongst these two physical-chemical processes the MAP process generates a more interesting end-product (i.e. struvite), allowing the recovery of both the main nutrients, i.e. nitrogen and phosphate, and moreover of magnesium.

2.3.4 H_2-based autotrophic nitrogen assimilation

Moving beyond nitrogen treatment in the form of complete dissipation into N_2 gas, H_2-oxidizing bacteria might represent an interesting way of recovering this nutrient by converting it directly into valuable microbial biomass. The direct use of hydrogen-oxidizing bacteria in the process of nutrients removal might create an interesting shortcut in the nitrogen cycle. The direct assimilation of reduced ammonia nitrogen by means of hydrogen-oxidizing bacteria would in fact avoid the irrational loop of oxidation-reduction of the already reduced nitrogen. In this case, by establishing a mixed culture of hydrogen-oxidizing bacteria alimented with additionally produced hydrogen (and oxygen), residual ammonium can be removed and converted into microbial biomass, which can be recovered and valorized. The costs of nitrogen removal following the hydrogen shortcut (see Table 2.1) are in our estimate a factor 2 higher than those of the other well-established biological processes for nitrogen dissipation (i.e. nitrification-denitrification) (see Table 2.3), nevertheless this re-synthesis approach might be suitable for applications such as upgrading residual ammonium to SCP for aquaculture systems. The latter systems typically suffer of inefficiency in terms of nitrogen input converted into harvestable product [108, 109]. Recent studies demonstrated the feasibility and the effectiveness of converting ammonia-nitrogen directly to microbial biomass via heterotrophic microbial metabolism [110]. In this approach, high C/N ratios are set, and the heterotrophically produced microbial biomass is used as additional food source by fish or shrimps [109]. The microbial biomass produced by the H_2-based autotrophic nitrogen assimilation might be used in the same way in aquaculture systems. Moreover, as previously mentioned, the ability of these bacteria to accumulate PHB might enrich the microbial biomass in prebiotic feed additives [36, 69].

2.3.5 Nitrogen removal and upgrading in the water factory

The aforementioned physical-chemical air-stripping and MAP processes might represent the starting line of a H_2-based biorefinery integrated in the water treatment plant, thus making it a "water factory". A possible process scheme based on nitrogen removal by air stripping or MAP and subsequent upgrade into added value microbial biomass is proposed in Figure 2.2.

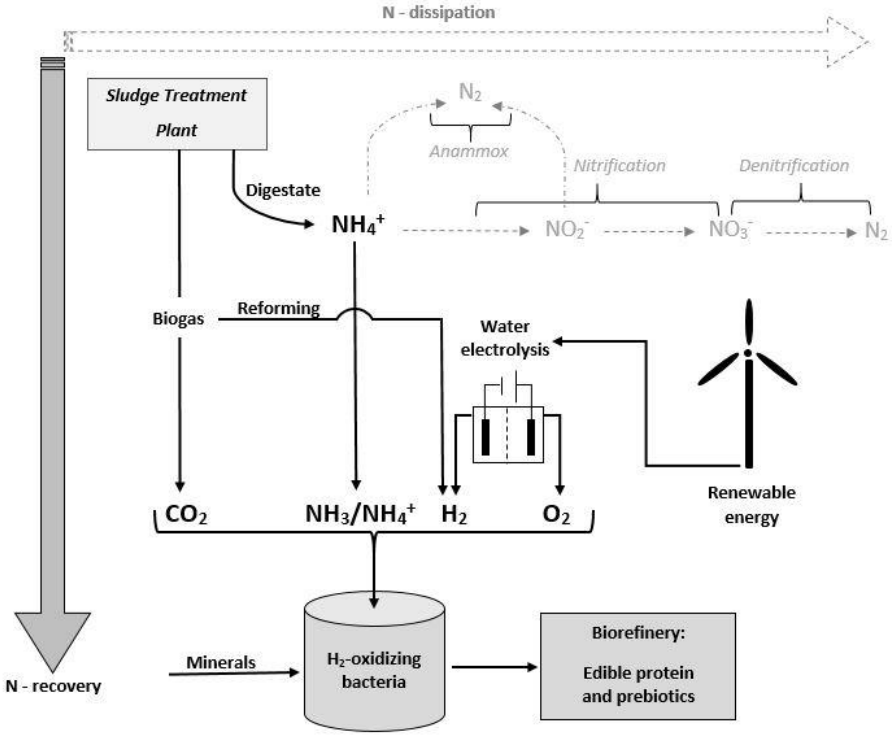

Figure 2.2. *Process scheme of the possible integration of H_2-based biorefinery within the used water factory. The gray characters and the dashed lines indicate the conventional approach of nitrogen removal by dissipation into N_2 gas.*

Nitrogen recovered from anaerobic digestate or reject water might be used as basic nutrient for the build-up of microbial proteins by means of H_2-oxidizing bacteria, fed with hydrogen and oxygen produced by renewable-energy-powered water electrolysis. Ultimately, this would connect the production of microbial by-products such as microbial biomass rich in proteins to resource recovery from used industrial or domestic water. Certainly, the use of nitrogen recovered at current costs of 6 Euro/kg N would sensibly increase the final cost of the produced biomass, yet these costs relate to total removal of ammonia. Aspects such as land scarcity for conventional crop production and new innovative technologies for recovering the easy to harvest part of

ammonia [111–113], certainly offer perspectives in the near future for such microbial route of upgrading nitrogen. The overall process of ammonium removal and re-integration in valuable microbial biomass would include also the major advantage of capturing excess of CO_2 from water treatment plant. Either using the CO_2 collected from the process of upgrading biogas to bio-methane [114–116], or the CO_2 emissions coming from biogas burning for heat and power generation, the implementation of a H_2-based biorefinery within water factory can thus decrease its environmental footprint in terms of greenhouse gas (GHG) emissions. Another intriguing aspect of this approach is the high volumetric productivity of the hydrogenotrophic bacteria. Cell concentrations up to more than 90 g CDW/L, with a maximum cell production rate of 5.23 g CDW/L·h and 5.02 g PHB/L·h where already obtained [67]. This offers the possibility to properly design reactor systems that would ultimately give a great advantage in terms of volumetric loading rate or area footprint. The most promising process line to be considered here is the renewable energy-to-hydrogen line, which involves electricity generation from renewable energy sources (wind, solar etc.) and electrolysis of water. This approach offers the major advantage of providing, in a sustainable way, both the hydrogen and the oxygen needed for the microbial bio-synthesis by hydrogen-oxidizing bacteria. Together with renewable energy, water electrolysis might be also powered by off-peak electricity, i.e. electrical power available when the energy demand is lower and which would be otherwise wasted. The fact that reforming technical modules are already operational on biogas, converting with high efficiency methane and CO_2 to H_2 is also of interest. The biogas might then be reformed to valuable hydrogen, with only few traces of CO (H_2/CO ratio around 0.97 [117]). This process has already been demonstrated during lab-scale tests as able to achieve biogas to hydrogen conversion efficiencies as high as 94-95% [117, 118]. Obviously, the key challenge will be the optimal design of the full scale reactor system assuring safety with respect to the used mix of gases (lower explosion limits for oxygen of 6.9% [67]) and the adequate harvesting and processing of the biomass in a way that the latter can be a source of one or more bio-based performance chemicals (SCP, PHB etc.)

2.4 Conclusions and future perspectives

As discussed in the present mini-review, the systematic and rational implementation of hydrogen-oxidizing bacteria, either as axenic cultures, or as evolving microbiomes, offers promising perspectives. Reconsidering the application of these microbial species within a broader and modern framework, matching them with the exploitation of green energies directed to hydrogen production, might give the opportunity to implement innovative process lines in the framework of resource recovery. This might help broadening the possibilities of mitigating the inefficiency and the environmental footprint of conventional used water treatment plants and related resource recovery systems. Powering the bio-refineries with hydrogen can also represents a step forward towards a more integrated and sustainable energy management within the urban context. Currently hydrogen is increasingly regarded as possible energy storage system in the so-called “power-to-gas” approach. There, the inherent instability of renewable energy production (mainly solar and wind energy) and excess of grid electricity (off-peak energy) is mitigated by the production of hydrogen by water electrolysis. The produced hydrogen gas is fed into the gas grid or converted to methane after methanation [119]. Furthermore, the hydrogen can be used as raw material for chemical, petrochemical, metallurgy and food industry [120]. Such hydrogen production systems can be of use also for upgrading the present WWTP to new “water factories”. In this context, hydrogen can serve as electron donor for many metabolic pathways in the broader context of hydrogen-utilizing microorganisms. Processes such as hydrogenotrophic denitrification for tertiary urban wastewater treatment for direct water reuse [121], as well as hydrogenotrophic sulphate reduction in sulphate-rich industrial wastewater [122] for recovery of valuable heavy metals by the produced biogenic hydrogen sulfide H_2S [123, 124], are examples of the broad possibilities offered by this kind of microorganisms in resource recovery. Hydrogen-oxidizing bacteria can, in the approach designed in this paper, be the primary users of such clean and valuable energy carrier. Plenty of technical challenges remain to be dealt with to come to effective upgrading of CO_2 and NH_4^+-N by means of H_2 and O_2. Aspects such as gas mass transfer and flammability of the gas mixture [125] can be tackled by employing rational and cost-effective combinations of the latest advances offered by process engineering [126, 127]. Moreover, future developments in used water treatment systems will soon provide other possibilities of matching resource

recovery with renewable energy production. In this framework, unexpected and intriguing new opportunities are emerging from research on electrochemical and bio-electrochemical systems (BES). Particularly, the generation of hydrogen coupled with the recovery of ammonia from anaerobic digestate [113], reject water [128] or urine [129] by means of electrochemical or BES system is a promising line to be followed. It might in fact represent an elegant platform for innovative used water treatment systems coupled to H_2-based biorefineries. In view of the increasing demand for quality feed and food protein, the realization of a H_2-based biorefinery might lead to the production of high quality feed and food at minimal land requirements. This concept will furthermore mitigate CO_2 emission and enhance the sustainability of existing and future water treatment plants. By coupling the conventional disintegrative capabilities of the microbes in general and of the methanogenic microbiome in particular, to a set of novel re-synthesis capabilities of the hydrogenotrophs, the water treatment factory can first generate useful building blocks such as ammonia, carbon dioxide and minerals which can be “accredited for re-use” and effectively upgraded to products desired by the consumer. In this way hydrogen-oxidizing bacteria can re-emerge as pivotal workhorses in processes aiming to the inversion from resource destruction to resource recovery and re-synthesis of valuable products from low value chemical constituents present in various streams of the current bio-economy.

2.5 Acknowledgments

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CHAPTER

3

ENRICHMENT AND CHARACTERIZATION OF HYDROGEN OXIDIZING MICROBIOMES

CHAPTER 3

ENRICHMENT AND CHARACTERIZATION OF HYDROGEN OXIDIZING MICROBIOMES

Abstract

The use of axenic cultures is an established practice in the biotech industry. Yet, different approaches might allow the use of consortia of microorganisms, offering good biotech performances without the need of stringent and costly sterility measures. In the present work, an environmental sample composed by a generic mixed microbial community was selectively enriched, eventually establishing a consortium of microorganisms able to thrive on hydrogen gas oxidation under autotrophic conditions. The effect of headspace oxygen levels on the biomass yield as well as on gas consumption was investigated, demonstrating its major influence on the hydrogen oxidation metabolism characterizing the biological system. Furthermore, the enriched culture was tested under different physico-chemical conditions in microtiter plate experiments, highlighting some important kinetic features. Finally, molecular analysis on the enriched microbial community revealed a strong balance between hydrogen oxidizing bacteria (HOB) and other bacteria within the established microbial consortium.

3.1 Introduction

Amongst the numerous studies on hydrogen-oxidizing bacteria, there is, to the best of our knowledge, no report of lithotrophic mixed microbial cultures established on hydrogen, oxygen and carbon dioxide. Neither is there information to what extent such microbial species can evolve together and become gradually evolved and organized to achieve maximum growth efficiencies and yields. The microbial characterization of single bacterial strains able to oxidize molecular hydrogen in presence of oxygen allowed to obtain a vast knowledge about their physiology and metabolism [60] and their kinetic parameters [61]. A major achievement in this line is the report on the genome sequencing of *Cupriavidus necator* H16 [62]. This allowed to reveal the microbial features that attracted the attention of researchers interested to explore the limits of specific bacterial strains under defined conditions. Nevertheless, the exploitation of axenic cultures in real scale applications faces problems of external contamination, and the measures to be taken often hamper scaling-up biological processes under such strictly sterile conditions. The exploitation of evolving microbial communities has one major disadvantage: generating a biomass whose composition cannot be assured to be constant. Yet, it offers several particular advantages. In contrast to axenic cultures, a non-specific biomass is easy to acclimate to different environments, not requiring any strict sterile environment to carry out its bioconversion [130, 131]. This more pragmatic approach is of vital importance when dealing with used water treatment and resource recovery. In this framework, when one considers a mixed microbial community adapted to a natural or artificial environmental niche, which has acquired a specific structure and metabolism, the term microbiome is appropriate [132]. Besides their ability to cope with rapid changes in environmental conditions [133], an important feature of microbiomes is the ability to restructure themselves when subjected to a selective pressure. Therefore, by applying strict environmental niche conditions such as the supply of H_2, O_2 and CO_2, a generic microbial community can be rapidly enriched with highly effective hydrogen-oxidizing bacteria. Following the same line of development established for mixed cultures of methane-oxidizing bacteria and heterotrophic bacteria, autotrophic hydrogen-oxidizing bacteria might also take advantage of autotrophic-heterotrophic interactions. A clear example of such advantage is reported for full-scale production of SCP from natural gas [95], with the methane-oxidizing bacteria accumulating excess of acetate in the

culture media. In that case, the shift from sterile to semi-sterile condition and the addition of heterotrophic bacteria allowed to remove metabolic by-products which were hampering the growth of autotrophic methane-oxidizing bacteria. This aspect has been recently elucidated also at lab scale: increased heterotrophic richness allowed to enhance the functionality of a methane-oxidizing bacteria in the same mixed microbial culture, with methane oxidation being the operational parameter [134]. In the case of hydrogenotrophic bacteria, different interacting microorganisms might become the backbone of a collaborating auto-heterotrophic microbiome able to use hydrogen and carbon dioxide and, moreover, ammonia with high efficiencies and without the need of strictly sterile conditions. The exploitation of such microbial community can allow, for instance, to recover ammonia from anaerobic digestate or reject water of municipal WWTP, upgrading all the simple components to valuable biomass rich in proteins and PHB.

The aim of this first experimental phase was to selectively enrich and characterize a mixed microbial community with H_2-oxidizing bacteria, able to autotrophically capture NH_4^+-N and CO_2 by using H_2 as electron donor and O_2 as electron acceptor.

3.2 Materials and methods

3.2.1 Enrichment of hydrogen-oxidizing bacteria

3.2.1.1 Microbial inoculum

Aerobic sludge from a local food (potatoes) processing plant (Nazareth, Belgium) was used as an initial mixed culture for the enrichment of autotrophic HOB community. The inoculum was characterized in terms of main physico-chemical parameters (CDW = 7 g/L; pH = 7.5; CODs = 126 mg/L; NH_4-N = 27 mg/L, PO_4-P = 30 mg/L, NO_2-N = 0,14 mg/L, NO_3-N = 0,44 mg/L) and stored at 4 °C before being used to start-up the experiment.

3.2.1.2 Reactor setup and operation

The enrichment was carried out in a 1 L gas fermentor. The reactor was connected to 3 gas bags supplying (total volume 20 L) a gas mixture composed by $H_2/O_2/CO_2$ with the following standard composition: 65/20/15 (vol/vol). Prior to use, each gas bag was

flushed with Alphagaz 2-grade H_2, O_2 and CO_2 gasses (Air Liquide, Belgium). The gaseous $H_2/O_2/CO_2$ atmosphere was constantly recirculated between the culture vessels and the gas bags by means of a peristaltic pump adapted to gas recirculation (Sci-Q 300, Watson Marlow, Belgium). The gas bags were all connected in series to the reactor, and each day 20 L of saline solution was constantly pumped into the gas bags to compensate for gas consumption by means of microbial activity. The unutilized gas was collected in a water column connected to the reactor, allowing quantifying the overall gas consumption. The column had a height of 1 m, therefore setting an overpressure of 100 mbar (10 kPa) into the reactor system. A scheme of the reactor setup is reported in Figure 3.1.

The reactor was placed in a 28 °C temperature controlled room and shaken at 150 rpm. A volume of 500 mL of mineral media inoculated with 10% of inoculum was used at start. The mineral medium was prepared accordingly to Yu et al. [59] for HOB isolation and culturing, having the following composition of macronutrients (1 L): 2.3 g KH_2PO_4, 4.0 g $Na_2HPO_4 \cdot 7H_2O$, 1 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $NaHCO_3$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.05 g ferric ammonium citrate, and 1 mL trace element solution. The trace element solution contained (1 L): 0.6 g H_3BO_3, 0.4 g $CoCl_2 \cdot 6H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.06 g $MnCl_2 \cdot 4H_2O$, 0.06 g $NaMoO_4 \cdot 2H_2O$, 0.04 g $NiCl_2 \cdot 6H_2O$, and 0.02 g $CuSO_4 \cdot 5H_2O$. The pH of the reactor was adjusted manually on a daily basis.

The first enrichment phase lasted about 40 days, during which the reactor was operated under batch conditions. The duration of each batch test was dependant on the growth of the biomass under the constant supply of $H_2/O_2/CO_2$ gasses. Biomass growth was followed by monitoring the increase of cell dry weight (CDW) over the course of the experimental run. When ammonium nitrogen was depleted below 10 mg/L, 50 mL of bacterial culture was withdrawn and diluted into 450 mL of fresh medium to restart the enrichment and select for HOB.

After constant and reproducible growth was obtained in the conditions above described, the culture was considered enriched, and the biomass yield as well as the gas consumption efficiency were studied under different oxygen concentrations by varying the composition of the gas mixture in terms of initial oxygen concentration. During this experimental phase, a solid retention time (SRT) of 7 days was set by withdrawing every day 70 mL of mixed liquor from the reactor, therefore allowing to operate the reactor under sequencing batch reactor mode (SBR).In this phase, biomass concentration and gas consumption were monitored in order to obtain an

indication of how the biomass yield was affected by different gas composition, and particularly by varying O2 levels. The measurements were carried out every 2 to 3 days.

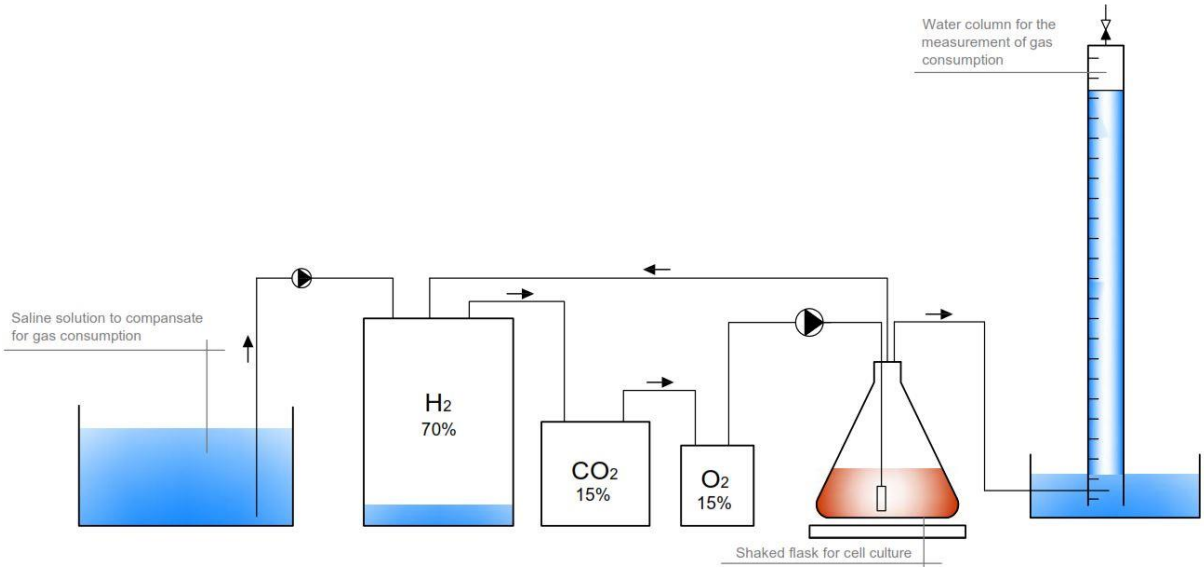

Figure 3.1. *Scheme of the experimental setup used for the HOB enrichment. Percentages of gasses indicate the relative volumetric concentrations, and varied along the course of the experiment.*

3.2.2 Analytical methods

NH_4^+-N concentrations were determined by means of cuvette tests (Hach Lange, range 0-47 mg NH_4^+-N/L). Cell Dry Weight (CDW) was measured in duplicate after water was evaporated at 105 °C for 24 h. Prior to analysis, the samples were centrifuged at 12500 g for 10 minutes for three times, each time re-suspending the biomass pellet in demineralized water.

3.2.3 Calculations

The overall gas was calculated by the subtracting the volume of gas collected in the water column to the overall volume of gas supplied by means of the 3 gas bags during the same time period, and defines as:

$$Overall\ gas\ consumption\ (\%) = \frac{Total\ gas\ supplied\ (L) - Total\ gas\ unutilized\ (L)}{Total\ gas\ supplied\ (L)} \times 100 \qquad (1)$$

With hydrogen gas as the electron donor for the HOB, the biomass yield on H_2 gas is expressed in terms of Chemical Oxygen Demand (COD) hydrogen gas equivalent. The yield is calculated as:

$$Y_{H_2}\left(\frac{g\ CDW}{g\ H_2-COD}\right) = \frac{CDW\ (g/L)}{H_2\ gas\ consumption\ (mol)\times 16\ (g\ COD/mol)} \times Liquid\ volume\ (L) \qquad (2)$$

Since no gas measurement could be done on the gas during this experimental phase, the H_2 gas consumption was calculated by assuming that the same volumetric composition of the gas fed to the reactor could be found in the gas collected in the water column. The final H_2 gas consumption was therefore calculated as:

$$H_2\ gas\ consumption\ \ (mol) = \frac{(Total\ gas\ supplied\ (L) - Total\ gas\ unutilized\ (L)) \times initial\ \%\ H_2\ gas}{22.5\ (L/mol)\ (at\ 28\ °C\ and\ 1.1\ atm)} \qquad (3)$$

3.2.4 Hydrogen oxidizing microbiome characterization

3.2.4.1 Microtiter growth experiments

A 10% solution of the enriched HOB culture was prepared by diluting 50 mL of the enriched culture at 4 g CDW/L into 250 mL of mineral medium. The 250 mL of culture was then placed into a gas washing bottle (OCHS, Germany). The gas washing bottle was continuously mixed by means of a magnetic stirrer (700 rpm) and connected to the same electrochemical cell used for the microtiter experiment (further described later). After the initial ammonium nitrogen concentration (500 mg NH_4-N/L) was used by the microorganism for biomass growth, achieving concentrations of 4-5 g CDW/L, a 0.5 % solution of the culture was prepared and used to inoculate the microtiter plate.

3.2.4.2 Experimental setup: electrochemical cell and microtiter plate

The oxygen/hydrogen mixture for growth of the hydrogen oxidizing microorganisms was provided by means of a lab scale electrolyser. The electrolyser consisted of a two-chamber Perspex reactor. Each chamber had a working volume of 128 ml (8*8*2 cm). The chambers were separated by a cation exchange membrane (CEM; membranes international, USA). An IrOx coated Ti mixed metal oxide electrode (Magneto, The Netherlands) was used as the anode and a stainless steel mesh was used as a cathode electrode. A 1L 0.5 M Na_2SO_4 solution in distilled water was used as the electrolyte in both compartments and refreshed every 7 days of continuous operations.

The electrolyte was recirculated over the electrode compartments at a rate of 1200 ml/h. An electrical current of 1.5 A (equivalent to theoretical H_2/O_2 gas flow rate of 20 mL/min) was applied by means of a power supply unit. The H_2/O_2 gas mixture was led through a bottle with 1 M $NaHCO_3$ to provide CO_2 to the gas mixture as a carbon source for the HOB. The $NaHCO_3$ solution was refreshed every 24 hours to prevent a pH increase leading to less efficient carbonation of the gas flow. The gas flow rate (water displacement method) and composition (Compact GC, The Netherlands) were measured before and after each microtiter growth experiment.

Growth experiments were conducted in a Tecan Sunrise microtiter plate reader (Tecan, Austria) placed in a 28 °C temperature controlled room. Off the shelf 96 well plates were fitted with gauge needles to provide gas in- and outlets. The plates were sealed with commercial available petroleum jelly to provide a gas tight incubation according to Geirnaert et al. [135]. A scheme of the experimental setup is reported in Figure 3.2.

Growth was measured as an increase in optical density at 620 nm (OD_{620nm}) every 15 minutes. All growth conditions were repeated as 6 replicates on each plate unless stated otherwise. No statistical elaboration was carried out on the collected data.

Given the mixed nature of the microbial culture, no relation could be established at this stage between optical density (OD) and colony-forming units (CFU/mL). The controls were prepared by using the same mineral medium without the inoculation step. A test was considered valid if the controls did not show any increase in optical density, thus confirming that no external or well-to-well contamination occurred within the microtiter plate. Controls were also repeated as 6 replicates on each plate.

Optical density data were converted into 8-point moving average values. Data on the hour was used for analysis of the growth curves by means of the software provided by Hall et al.[136]. This allowed converting the optical densities observed in specific growth rates and individuating the maximum specific growth rate associated to each different tested condition.

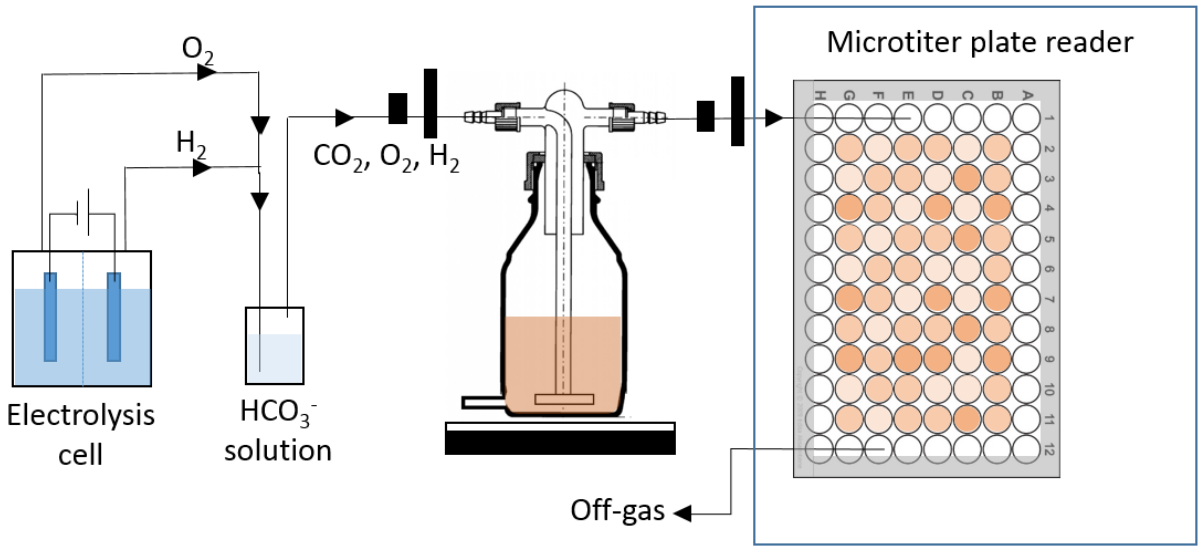

Figure 3.2. *Scheme of the experimental setup used for the HOB kinetic experiment. The 96 well microtiter plate was sealed and the same gas mixture fed to the gas culturing bottle was sparged over the wells of the microtiter plate.*

3.2.4.3 Microbial community analysis

A 50mL sample from the enriched culture was collected for 16S rRNA Illumina sequencing analysis. The sample processing and analysis were carried out in an external laboratory (Microbial Insights, USA).

3.3 Results

3.3.1 Preliminary screening of biomass yield and gas utilization efficiency

After about 6 weeks of sequencing batch operations the mixed microbial culture displayed constant and reproducible growth, allowing to consider the microbial culture enriched in hydrogen-oxidizing bacteria. The enriched microbial community was then cultured under varying oxygen levels and the efficiency of the process was monitored in terms of biomass yield and gas consumption efficiency. Biomass yield was monitored by measuring the cell dry weight (CDW) produced per gram of hydrogen COD (H_2-COD). The main results observed along the 120 days of tests are resumed in Figure 3.3.

When during the first 30 days of operations the oxygen level was set at 20% (vol./vol.), the biomass yield fluctuated between 0.02 and 0.13 g CDW/g H_2-COD, whereas the gas consumption efficiency increased constantly from the initial 50% to the maximum

of 100% after 20 days of operations. Subsequently to lowering the oxygen levels between 7 and 4% (vol./vol.) between day 30 and 85, the biomass yield increased substantially, ranging between 0.09 and 0.29 g CDW/g H_2-COD, with the highest values obtained when the oxygen levels were lowered to 4%. During this experimental period the gas consumption was sensibly lower, with average values around 20 to 30%. The final experimental phase (from day 86 to 120) was characterized by a constant oxygen level of 13%. During this period the biomass yield varied between 0.09 and 0.21 g CDW/g H_2-COD, whereas gas consumption was between 30 and 56%.

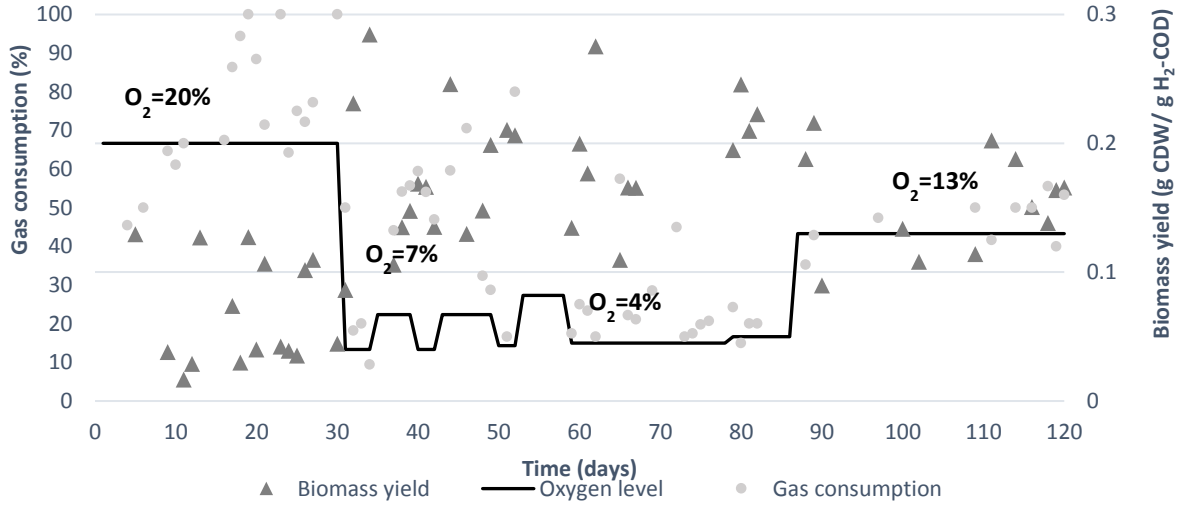

Figure 3.3. *Biomass yield and gas consumption observed with different oxygen levels over 120 days of reactor operations.*

3.3.2 Kinetic characterization

A preliminary kinetic characterization allowed to screen the influence of varying nitrogen source, pH and bicarbonate concentration on the specific maximum growth rate of the hydrogen oxidizing microbiome. The results are reported in Figure 3.4.

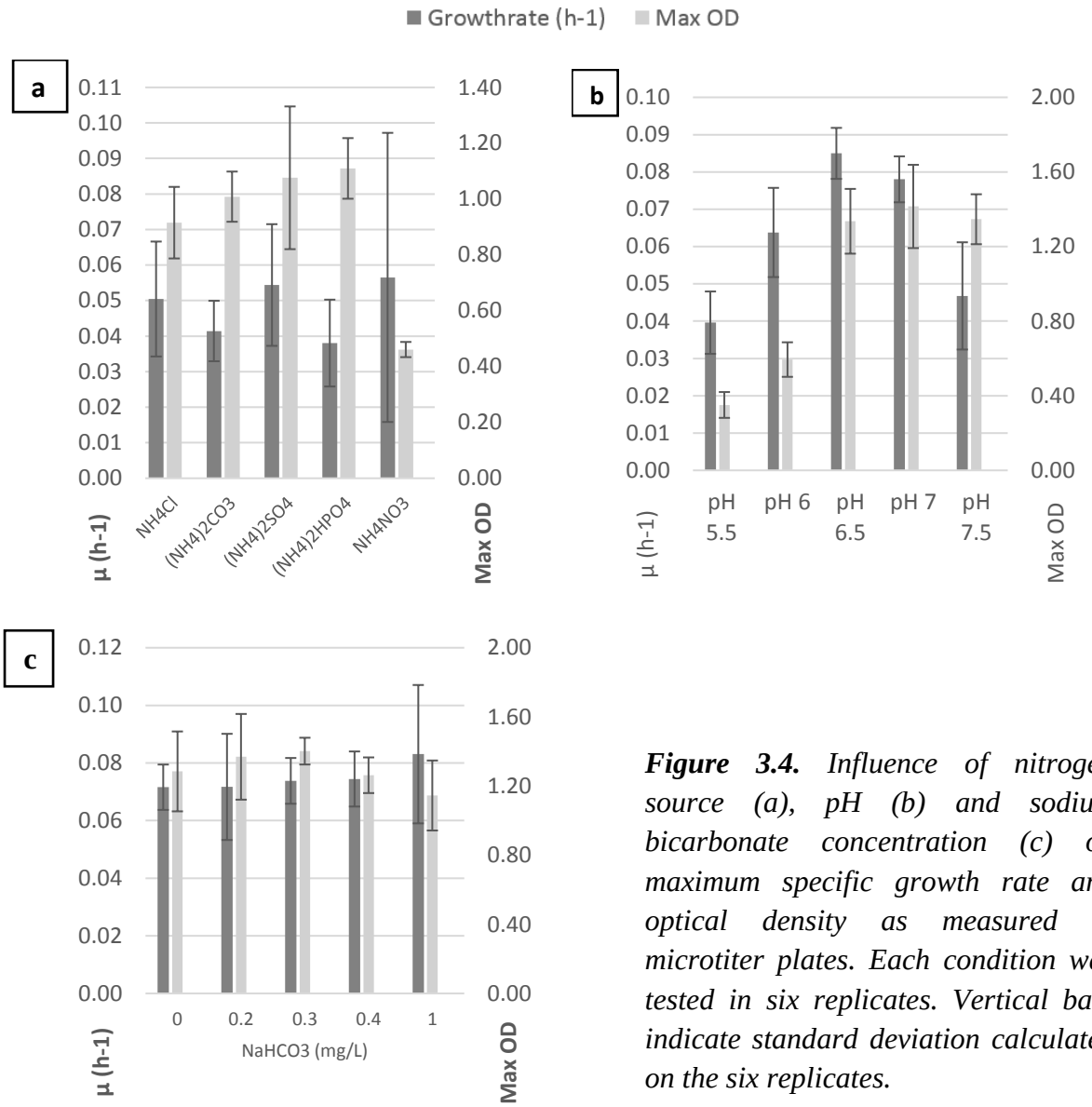

Figure 3.4. *Influence of nitrogen source (a), pH (b) and sodium bicarbonate concentration (c) on maximum specific growth rate and optical density as measured in microtiter plates. Each condition was tested in six replicates. Vertical bars indicate standard deviation calculated on the six replicates.*

In terms of nitrogen source (Figure 3.4a), ammonium chloride and ammonium sulphate allowed the highest maximum specific growth rate of 0.05 and 0.054 h^{-1}. The use of ammonium nitrate also allowed evolving a maximum specific growth rate of 0.057, yet the latter value resulted affected by a high standard deviation (±0.04 h^{-1}), therefore not allowing to consider such value as representative. Moreover, the maximum optical density achieved was only about 50% of the maximum values achieved with the other nitrogen sources.

The influence of pH is reported in Figure 3.4b. The highest maximum specific growth rate of 0.085 h^{-1} was observed with a pH of 6.5, which decreased to 0.078 and 0.063 h^{-1} when pH was 7 and 6, respectively. Lower values of 0.046 and 0.039 h^{-1} were measured when pH was set at 7.5 and 5.5, respectively.

The variation of sodium bicarbonate concentration between 0 and 1 g/L (Figure 3.4c) did not result in noticeable changes of the maximum specific growth rate, which ranged between 0.7 and 0.82 h^{-1}.

3.3.3 Microbial analysis

A first screening of the microbial composition of the HOB microbiome was carried out by means of Illumina sequencing, the results are summarized in Figure 3.5. The microbial community showed a diverse composition, and amongst the 12 genera identified, *Ancylobacter* [137], *Xanthobacter* [138], *Hydrogenophaga* [139] and *Arcobacter* [140] have been documented as able to autotrophically grow on hydrogen, oxygen and carbon dioxide. The latter constitutes about 47 % of the quantitatively detected genera distribution in the HOB microbiome. No direct evidence of aerobic hydrogen oxidation is available in literature for the other genera.

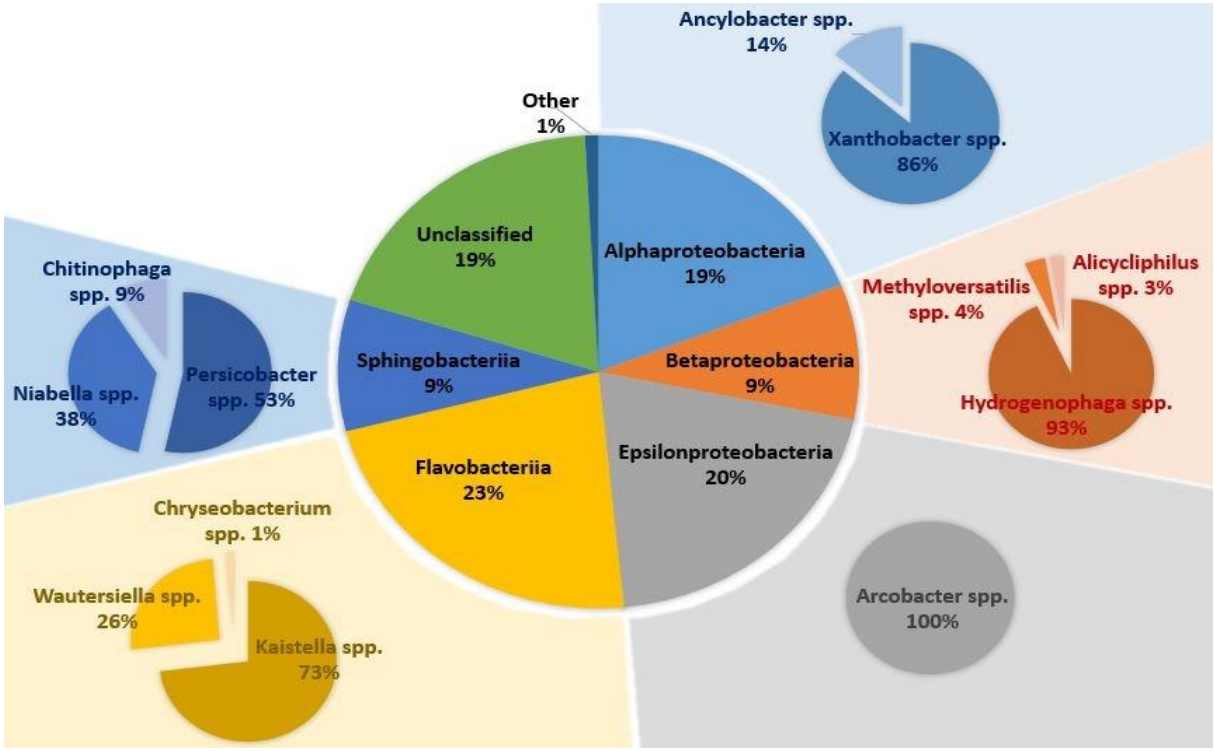

Figure 3.5. *Phylogenetic composition of the HOB microbiome assessed by NGS Illumina MiSeq (2x250bp). The central graph resumes the percentage of each class within the microbiome. Each class is then characterize in terms of genera composition (external graphs).*

3.4 Discussion

This first experimental phase allowed to have the proof of concept that a mixed culture can be enriched in H_2-oxidizing bacteria, able to capture NH_4^+-N and CO_2 into new cell material.

After the enriched culture displayed a stable growth, the variation of the oxygen levels revealed its high impact on the efficiency of the biological process, both in terms of biomass yield and gas consumption. Interestingly, biomass yields were inversely proportional to oxygen levels, whereas gas consumption was directly proportional to oxygen levels. This is in accordance with Ju et al. [141], where increasing levels of oxygen resulted in lower biomass yield but higher gas consumption, whereas lower oxygen levels corresponded to higher biomass yields and concomitant low gas consumption. This is explained by the same authors by relating it to a general principle of thermodynamics, accordingly to which the higher is the efficiency of the reaction, the slower the reaction will proceed. In microbiological terms this has been possibly attributed to proton leakage, decoupling of ATP synthesis from the proton-motive force or to the competition of CO_2 and O_2 for the Rubisco enzyme. The latter, known to display an additional oxygenase activity, could cause the oxygen molecule to compete with CO_2 for the enzyme-bound eno-diolate of RuBP, which reacts with RuBP to form 3-phosphoglycerate and phosphoglycolate [141]. Besides representing an interesting research question, the latter aspect poses a technical challenge in the sense that high efficiencies, beneficial for the overall process, are matched by low rates.

Besides the importance of oxygen levels, the tentative kinetic characterization carried out in the microtiter plate, allowed to confirm some other known features of HOB, also displayed by the enriched mixed culture. In terms of nitrogen source, negligible differences was measured amongst the different sources tested, except for the low OD achieved in presence of NH_4NO_3, which requires further investigations. The pH sensitivity of the enriched community is also in accordance with other studies which proved single strains of HOB to have optimal growth rates within a restrict pH range of 5.5-7.5 [64]. Finally, the variation of initial bicarbonate levels did not influence the maximum growth rate of the biomass, suggesting that the supply of gaseous CO_2 remains crucial for the kinetics of HOB, as it was also observed by Ju et al. [141].

Finally, the microbial analysis composition displayed a high diversity within the enriched culture. More specifically, the relative abundance of HOB and other bacteria

was remarkable, revealing a surprising equilibrium achieved within the biological system between primary producers (HOB) and secondary consumers (other heterotrophic bacteria).

3.5 Conclusions

Enriching a generic microbial community in HOB was possible after only few weeks of operations, confirming how ubiquitous hydrogen oxidation is within bacteria. The establishment of a rather diverse microbial community, composed based on 16s ribosomal DNA estimates, by about 50% of HOB and 50% of other bacteria did not alter the overall metabolic features usually observed in single strains of HOB. In fact, biomass yield and gas consumption displayed the same dependency to oxygen observed with other known single HOB, whereas the kinetic behaviour with varying N-sources, pH and bicarbonate concentrations were comparable to other studies.
Overall, the preliminary enrichment allowed to demonstrate that hydrogen oxidation was the main driver of the mixed community. Based on such enrichment, further experiments were subsequently carried out in order to characterize more in depth the kinetic aspects as well as the biotech potentials of HOB.

3.6 Acknowledgments

We thank Dr. Jan Arends for the assistance and scientific guidance provided during the experiments with the microtiter plates.

Chapter 4

Autotrophic nitrogen assimilation and carbon capture for microbial protein production by a novel enrichment of hydrogen-oxidizing bacteria

Chapter redrafted after:

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CHAPTER 4

AUTOTROPHIC NITROGEN ASSIMILATION AND CARBON CAPTURE FOR MICROBIAL PROTEIN PRODUCTION BY A NOVEL ENRICHMENT OF HYDROGEN-OXIDIZING BACTERIA

Abstract

Domestic used water treatment systems are currently predominantly based on conventional resource inefficient treatment processes. While resource recovery is gaining momentum it lacks high value end-products which can be efficiently marketed. Microbial protein production offers a valid and promising alternative by upgrading low value recovered resources into high quality feed and also food. In the present study, we evaluated the potential of hydrogen-oxidizing bacteria to upgrade ammonium and carbon dioxide under autotrophic growth conditions. The enrichment of a generic microbial community and the implementation of different culture conditions (sequencing batch resp. continuous reactor) revealed surprising features. At low selection pressure (i.e. under sequencing batch culture at high solid retention time), a very diverse microbiome with an important presence of predatory *Bdellovibrio* spp. was observed. The microbial culture which evolved under high rate selection pressure (i.e. dilution rate $D=0.1h^{-1}$) under continuous reactor conditions was dominated by *Sulfuricurvum* spp. and a highly stable and efficient process in terms of N and C uptake, biomass yield and volumetric productivity was attained. Under continuous culture conditions the maximum yield obtained was 0.29 g cell dry weight per gram chemical oxygen demand equivalent of hydrogen, whereas the maximum volumetric production rate peaked 0.41 g cell dry weight per litre per hour at a protein content of 71%. Finally, the microbial protein produced was of high nutritive quality in terms of essential amino acids content and can be a suitable substitute for conventional feed sources such as fishmeal or soybean meal.

4.1 Introduction

Primary producers - autotrophic microorganisms - are essential for carbon and nutrients cycling. While fixing inorganic CO_2 into organic biomass they recycle nutrients (N and P) and provide food for higher life forms [142]. Primary producers such as microalgae and autotrophic bacteria can serve as alternative protein source in the form of microbial protein (MP) for livestock but also for human consumption [6, 143, 144]. Besides protein, microbes can also accumulate considerable amounts of biocompatible prebiotics such as PHB [37], thereby enhancing the nutritional value of the microbial biomass.

After being extensively studied in the past, mainly as means to upgrade fossil fuel (e.g. paraffin, natural gas) to protein supplements [8], the use of bacteria for microbial protein (MP) production has nowadays re-gained significant interest [145, 146] with natural gas based MP production entering the market economy [39]. Innovative approaches implementing bacteria to produce MP within the context of resource recovery from used water have also been recently proposed [14, 45, 148]. Indeed, the production of MP can allow the up-cycling of nitrogen and carbon dioxide recovered from used water streams, converting them into protein-rich feed and food substances. Different physico-chemical techniques can be implemented in the recovery of N and C substrates. Air stripping or pervaporative processes can recover N from concentrated streams such as anaerobic digestate, whereas pressure swing adsorption (PSA) can concentrate CO_2 from biogas, thus providing the building blocks which are at the base of MP biosynthesis.

Among the various metabolic pathways suitable for MP production, including both eukaryotic and prokaryotic microorganisms [6], autotrophic hydrogen-oxidizing bacteria (HOB) constitute a special and thus far unexplored metabolic niche with potential for novel applications in resource recovery and upgrade. Even if ubiquitous, autotrophic HOB have only received limited attention, with previous studies focusing on the use of axenic cultures comprising bacteria such as *Alcaligenes eutrophus*, *Ralstonia eutropha, Seliberia carboxydohydrogena* [56, 63, 76]. The metabolic features of autotrophic HOB allow them to grow on hydrogen (electron donor) and oxygen (electron acceptor) while fixing carbon dioxide into cell material and assimilating nitrogen into high quality protein [62, 149]. MP produced by autotrophic HOB is characterized by all the essential amino acids, having an amino acid profile

closer to high-quality animal protein rather than to vegetable protein [76]. Given this interesting feature, autotrophic HOB were already proposed as possible protein source within biological life support systems for space missions [150], as well as for human and animal nutrition [76].

An attractive characteristic of MP production with autotrophic HOB is the possibility to exploit the increasing potential of renewable energy generation. A clear example is the use of hydrogen gas produced from water electrolysis, powered by e.g. wind or solar energy, or also from biomass gasification [151] Recently, biomethane has also been proposed as possible renewable feedstock for hydrogen production by means of a combined heat, hydrogen and power generation unit (CHHP) [152, 153]. The possibility to implement such technologies on-site and produce hydrogen on demand might enable the direct up-cycling of mineral nitrogen and carbon dioxide recovered from wastewater treatment plants, as previously mentioned. Moreover, upcoming technological developments and the decrease of hydrogen prices [154] justify further research efforts towards the application of autotrophic HOB within resource recovery and up-cycling.

In the present study, we aimed to experimentally determine the feasibility of nitrogen and carbon upgrade into MP by means of a microbial community enriched in HOB using a lab-scale gas. Along the experimental investigation different culture conditions were imposed to the enriched HOB culture (i.e. sequencing batch and continuous). This was done in order to establish how the microbial community was shaped by the process conditions and how this affected the overall biological performance of the system, aiming at maximizing MP production (i.e. biomass yield and volumetric productivities). Nitrogen under the form of ammonium salt and gaseous CO_2 represented the N and C substrates needed for the production of MP protein by means of autotrophic HOB. The study started with the enrichment of a generic aerobic microbial mixed culture with autotrophic HOB under sequencing batch reactor operations. Consequently, the enriched mixed community was cultured in a continuous reactor configuration, resulting in the ongoing evolvement of a highly specific bacterial culture dominated by the genus *Sulfuricurvum.* The efficiency of the process in terms of gas utilization and by-product formation was monitored along the time course of the selective enrichment process. The microbial community analyses of the HOB microbiome under batch and continuous culture systems allowed delineating the evolution of the mixed bacterial community towards a quasi-monoculture dominated

by *Sulfuricurvum* spp. Finally, the MP produced was characterized in terms of crude protein content and amino acid profile in order to assess its nutritional value.

4.2 Material and Methods

4.2.1 Reactor operations and controls

A completely stirred tank reactor (CSTR) (Biostat A plus, Sartorius, Belgium) was used during batch as well as continuous experiments. The 5 L glass vessel, with a working volume of 3 L, was stirred at 700rpm with a 3-blade segment impeller to ensure completely mixed conditions. Hydrogen gas was produced on site by means of a lab-grade hydrogen generator (Alphagaz™ Flo H_2, Air Liquide, Belgium), while CO_2 from gas bottles was of the same grade of the one used during the initial enrichment of the culture. Compressed air was used to provide the oxygen. Gases were fed separately by means of 3 micro-spargers (Sartorius, Belgium) submerged in the reactor. Gas flows were monitored using gas rotameters (Omega, USA) and kept at H_2: 120 mL/min; CO_2: 25 mL/min; Air: 180 mL/min. The gas collected in the headspace was constantly recirculated by means of a peristaltic pump adapted to gas recirculation (Sci-Q 300, Watson Marlow, Belgium) using a fourth micro-sparger. Utilized gas by the bacteria, was bubbled through an external water lock (imposing an overpressure of 20 mbar) and subsequently vented to the atmosphere by means of a fume hood. Temperature and pH were automatically controlled and kept at 35±1 °C and 6.7, respectively.

4.2.2 Microbial inoculum

The enriched HOB microbiome obtained at the end of the work described in chapter 3 was used as start-up inoculum for the work described in the present chapter.

4.2.3 Sequencing batch and continuous reactor culture systems

Sequencing batch reactor (SBR) tests were started by transferring 300 mL of fully grown bacterial culture into 2.7 L of fresh mineral medium, allowing an initial cell dry weight Cell Dry Weight (CDW) concentration of 300 to 500 mg CDW/L. Each sequencing batch test was allowed to evolve for an average of 5 to 6 days before transferring the culture into fresh medium, corresponding to a solid retention time (SRT) of 6±0.5 days. Additional NH_4Cl was added to the standard mineral medium

composition in order to achieve initial NH_4^+-N concentration of 1.2 g/L. The sequencing batch culture was monitored along a period of 5 months.

Continuous reactor (CR) operations were set by supplying fresh media with a diaphragm pump (Qdos, Watson Marlow, Belgium), totalling a flow of 7.2 L/day. In the same way, 7.2 L/day of cell culture were constantly withdrawn from the CSTR reactor by means of a similar pump. The complete absence of biomass recirculation set hydraulic and (SRT) of 10 h. Under these continuous reactor conditions (chemostat), only bacteria with a specific growth rate "μ" equal or higher than the dilution rate D=0.1 h^{-1} could avoid being washed-out from the biological system. The continuous system was operated uninterruptedly for 3 months.

4.2.4 Analytical methods

NH_4^+-N concentrations were determined by means of cuvette tests (Hach Lange, range 0-47 mg NH_4^+-N/L). Cell Dry Weight (CDW) was measured in duplicate after water was evaporated at 105 °C for 24 h. Prior to analysis, the samples were centrifuged at 12500 g for 10 minutes for three times, each time re-suspending the biomass pellet in demineralized water. Gas samples collected from the reactor headspace were analyzed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (O_2, H_2 and N_2) and a Rt-Q-bond pre-column and column (CO_2). Concentrations of gases were determined by means of a thermal conductivity detector.

4.2.5 Analysis and characterization of microbial protein

Kjeldahl nitrogen content of the microbial biomass was analyzed according to Standard methods [155]. Organic nitrogen was determined as the difference between Kjeldahl nitrogen and ammonium nitrogen. The final protein content of CDW was obtained by multiplying the obtained value by applying a conversion factor of 6.25 as done in previous studies [156]. The dietary amino acids composition of the microbial biomass was determined by an external accredited commercial laboratory (Eurofins Denmark A/S, Denmark).

4.2.6 Microbial community analysis

Liquid samples for total DNA extraction were centrifuged for 10 min at 10000 RPM. Subsequently, the supernatant was removed and biomass pellet was stored immediately at −20◦C until further analysis following a protocol adapted from VilchezVargas et al. [157]. Cells were lysed with 1 mL lysis buffer (100 mM Tris/HCl pH 8.0, 100 mM EDTA pH 8, 100 mM NaCl, 1% (m/v) polyvinylpyrrolidone and 2% (m/v) sodium docecyl sulphate) and 200 mg glass beads (0.11 mm, Sartorius) in a FastPrepR - 96 instrument (MP Biomedicals, Santa Ana, USA) for two times 40 s (1600 rpm). After removing glass beads by centrifugation (5 min at 10000 RPM), DNA was extracted from supernatant following a phenol–chloroform extraction. DNA was precipitated with 1 volume ice-cold isopropyl alcohol and 0.1 volume 3 M sodium acetate for at least 1h at −20◦C. After removal of isopropyl alcohol by centrifugation (30 min, maximum speed), the DNA pellet was dried and re-suspended in 100 µL 1× TE (10 mM Tris, 1 mM EDTA) buffer. After finishing the extraction protocol, the DNA samples were immediately stored at −20◦C until further processing. Quality of DNA samples was analyzed by 1% (w/v) agarose (Life technologies, Madrid, Spain) gel electrophoresis. The PCR amplicons were purified with the innuPREP PCR pure kit (Analytik Jena, Jena, Germany), and sequenced with the primers used for PCR. 16s rRNA Illumina and Sanger sequencing analyses were conducted for each sample in triplicate by external commercial laboratories (Analytik Jena, Jena, Germany).

4.2.7 Calculations

The gas conversion efficiency was calculated as:

$$Gas\ conversion\ efficiency\ (\%) = \frac{Gas\ inlet\ (mol/min) - Gas\ outlet\ (mol/min)}{Gas\ inlet\ (mol/min)} \times 100 \qquad (1)$$

With hydrogen gas as the electron donor for the HOB, the biomass yield on H_2 gas is expressed in terms of Chemical Oxygen Demand (COD) hydrogen gas equivalent. The yield is calculated as:

$$Y_{H_2}\left(\frac{g\ CDW}{g\ H_2 - COD}\right) = \frac{CDW\ (g/L)}{H_2\ gas\ uptake\ (mol) \times 16\ (g\ COD/mol)} \times Liquid\ volume\ (L) \qquad (2)$$

The biomass yield on carbon dioxide is calculated as:

$$Y_{CO_2}\left(\frac{g\ CDW-C}{g\ CO_2-C}\right) = \frac{CDW\ (g/L) \times 0.5\ (g\ C/g\ CDW)}{CO_2\ gas\ uptake\ (mol) \times 12\ (g\ C/mol)} \times Liquid\ volume\ (L) \quad (3)$$

The mineral nitrogen upgrade efficiency is calculated as:

$$N\ upgrade\ effieicncy\ (\%) = \frac{NH4-N\ in\ (g/L) - X-N\ out\ (g/L)}{NH4-N\ in} \times 100 \quad (4)$$

Where *NH_4-N in* indicates the amount of NH_4-N fed to the reactor, respectively to the SBR and the CR systems and *X-N out* indicates the amount of dissolved nitrogen under the form of NH_4^+, NO_2^- or NO_3^- at in at the end of each SBR test and in the effluent of the CR system.

4.3 Results

4.3.1 Sequencing batch and continuous reactor performances

The enriched HOB culture was first cultivated under sequencing batch reactor (SBR) conditions, with a SRT of 6±0.5 days. The same experimental setup was then adapted to grow the HOB culture under continuous reactor (CR) configuration, imposing a SRT of 10 h. The main parameters analyzed both under SBR and CR configurations were: volumetric productivities (g CDW/L·h), biomass yields on hydrogen (g CDW/g COD-H_2) and carbon dioxide (g CDW-C/g CO_2-C) and hydrogen gas conversion efficiencies (%), as shown in Table 4.1.

Table 4.1. *Parameters of HOB cultivation obtained under SBR tests (averaged over three different sequencing batch tests) and CR operations (over 90 days of continuous operations). Maximum values were calculated for each batch for the data points which maximized volumetric productivity and biomass yield, whereas average values were calculated over the whole period.*

Parameter		**Sequencing Batch reactor**	**Continuous reactor**
Volumetric productivity (g CDW/L·h)	Average	0.078 ± 0.012	0.375 ± 0.015
	Maximum	0.187 ± 0.045	0.406
Y_{H2} (g CDW/g COD-H_2)	Average	0.073 ± 0.007	0.280 ± 0.010
	Maximum	0.157 ± 0.037	0.290
Y_{CO2} (g CDW-C/g CO_2-C)	Average	0.153 ± 0.023	0.427 ± 0.013
	Maximum	0.246 ± 0.058	0.456
H_2 gas conversion efficiency	Average	65% ± 4%	81% ± 2%
	Maximum	71% ± 3%	87%
N upgrade efficiency	Average	100%	87% ± 4%
	Maximum	100%	97%
Protein content (%CDW)	Average	66% ± 5%	71% ± 5%
	Maximum	73%	76%

The average values for each individual SBR test reported in Table 4.1 were calculated, by considering the initial and final point of each test over the duration of the experimental run (i.e. t=0 to t=120-144 h). Three subsequent SBR experimental run (t=41, 82, 120 days) were averaged together to summarize the values obtained along the SBR cultivation period. Maximum values indicate the maximum single data point measured during each individual SBR test For the CR operations, samples were taken for analysis three times per week over a period of 90 days (n=35). The average values reported in Table 4.1 show the average of the total amount of samples taken.

Under SBR conditions, an average volumetric productivities of 0.078 ± 0.012 g CDW/L·h was achieved. The latter value increased about 5-fold under CR configurations, reaching an average of 0.375 ± 0.015 g CDW/L·h. Biomass yields in terms of g CDW/g COD-H_2 increased from 0.073 ± 0.007 to 0.280 ± 0.010 g CDW/g COD-H_2, when changing from a SBR to a continuous operation mode. In the same way, CO_2–based yield increased from the minimum of 0.153 ± 0.023 g CDW-C/g CO_2-C observed during SBR cultivation to the maximum of 0.427 ± 0.013 g CDW-C/g CO_2-C. Hydrogen gas was also converted more efficiently when the reactor operated

continuously, with an increase of 16% compared to SBR operations, reaching 81 ± 2%. Maximum values observed under CR were almost double than observed under SBR conditions. A different trend was observed for the nitrogen upgrade efficiency. SBR conditions allowed the complete conversion of the ammonium nitrogen supplied into MP, which reached an average of 65 ± 5% of the microbial biomass CDW. Under CR operation, instead, about 13% of the total mineral ammonium nitrogen supplied was still present in dissolved form in the CR effluent, whereas the average protein content of the produced biomass was 71 ± 5% (%CDW).

4.3.2 Microbial community analysis

In order to assess the composition of the microbial community, DNA samples from the SBR (after 120 days of operations) and from the CR configurations (after 20 days of operations) were analyzed by means of 16S rRNA Illumina sequencing. The results are summarized in Figure 4.1.

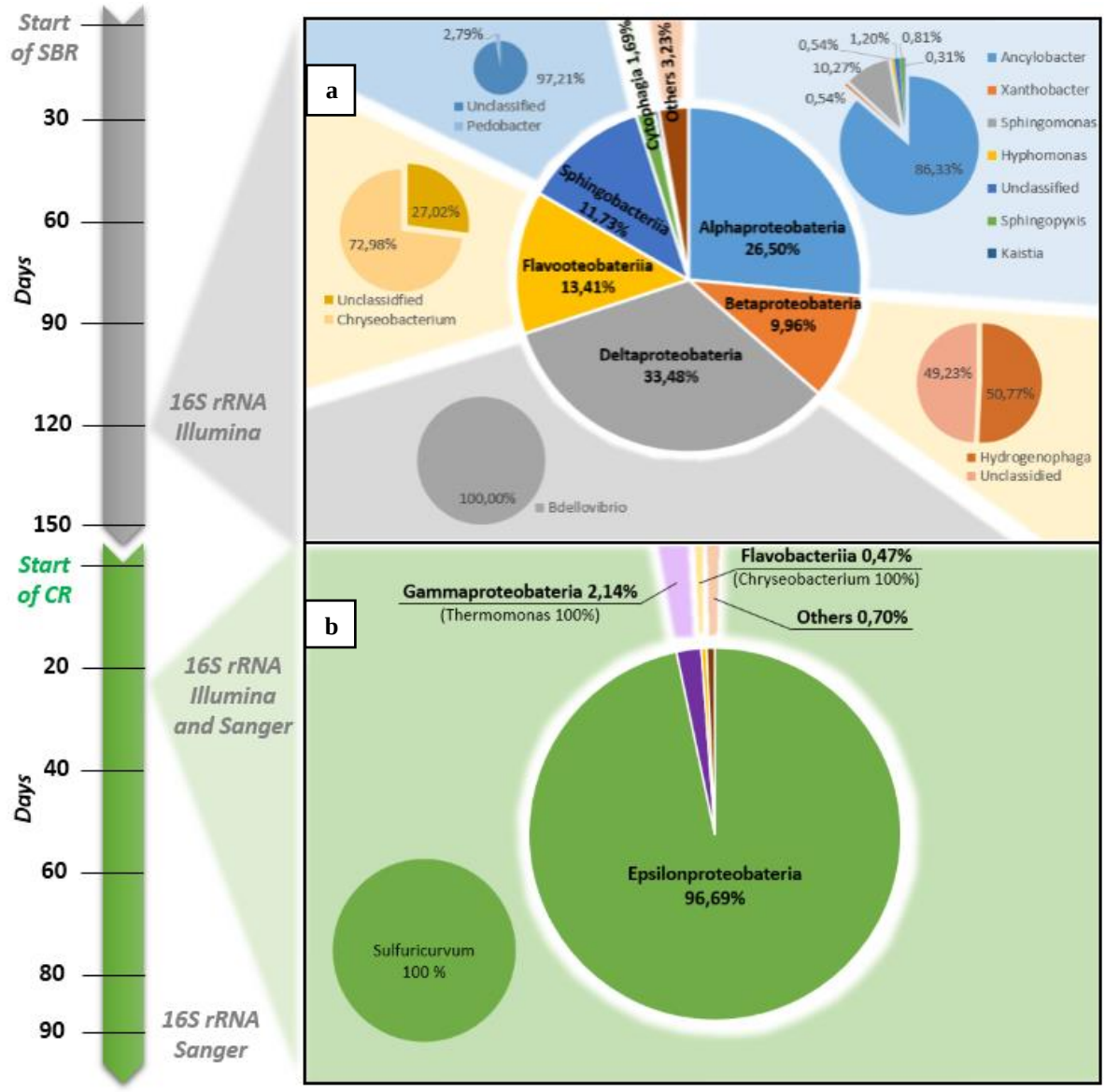

Figure 4.1 *Phylogenetic composition of the HOB microbiome during SBR (a) and CR (b) operations, assessed by 16S rRNA Illumina sequencing. The timeline indicates the duration of each phase: SBR and CR, and when DNA samples were processed for 16S rRNA Illumina and Sanger sequencing. The central graph resumes the percentage of each class within the microbial community. Each class is then characterize in terms of genera composition in the external graphs (a, b) or within brackets (b).*

The enriched microbial community cultivated under SBR conditions was characterized by a rather high diversity. Amongst the 12 genera identified, *Ancylobacter* [158], *Xanthobacter* [138] and *Hydrogenophaga* [139] have been already documented as able to carry out autotrophic oxyhydrogen metabolism. The latter constitute less than one-third of the quantitative genera distribution of the microbial community. No direct evidence of aerobic hydrogen oxidation is available in literature for the other genera present. Notably, the microbial community was dominated (one-third of the whole

quantitative genera distribution) by *Bdellovibrio*, a genus of the class of *Deltaproteobacteria* encompassing predatory bacteria able to invade and lyse various other Gram-negative bacteria [159]. The remaining genera detected (about 30% relative abundance) were mainly composed by the classes of *Falvobacteriia* and *Sphingobacteriia*, known as aerobic chemoorganotrophic bacteria [160, 161].

Following the SBR cultivation period, the effect of the first 20 days of CR operations on the microbial community was investigated by means of a second 16S rRNA Illumina sequencing analysis. As shown in Figure 4.1b, the simple implementation of high rate ($D=0.1\ h^{-1}$) continuous reactor operations led to a remarkable selection within the microbial community, with almost 97% of the total community composed by a single genus: *Sulfuricurvum*. Almost 80% of the remaining 3% was composed by only other two genera: *Gammaproteobacteria* (*Thermomonas*) and *Flavobacteriia* (*Chryseobacterium*).

The DNA sample used for the 16S rRNA Illumina sequencing analysis of the CR was subsequently analyzed by means of 16S rRNA sequencing, together with a second sample taken after 90 days of continuative CR operations. The latter was done in order to confirm the stability of the microbial community composition and to gain more in depth information on the dominating *Sulfuricurvum* genus. For both samples the analysis indicated similarities at the level of 98 and 99% to *Sulfuricurvum kujiense* strains *YK-2*, *YK-3* and *YK-4*, as well as to other uncultured *Epsilonproteobacteria* when compared using NCBI BLAST under default settings [162].

4.3.3 Protein and amino acid profile

The bacterial biomass grown under CR configurations was harvested at day 90 (i.e. at the end of the CR cultivation period) and analyzed for crude protein content as well as for essential amino acids composition.

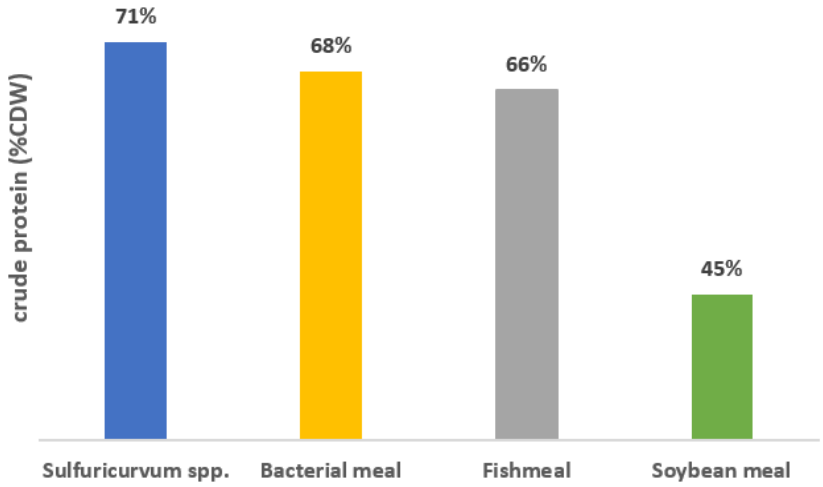

Figure 4.2. *Crude protein content on CDW basis of the microbial biomass produced under CR configuration by the Sulfuricurvum spp. dominated culture (this study) compared with other microbial protein (bacterial meal), animal protein (fishmeal) and vegetable protein (soybean meal)* [9].

Figure 4.2 compares the results obtained in this study with reference protein feed additives such as fishmeal, soybean meal and bacterial meal. The latter is a MP product obtained from methane oxidizing bacteria (*Methylococcus capsulatus* grown in association with other heterotrophic bacteria) already produced at pilot scale and tested in several feed trials involving monogastric animals as well as aquaculture species, for which the EU already approved the use in animal nutrition [9]. Fishmeal and soybean meal were chosen as a reference for animal and vegetable protein, respectively. Bacterial meal allows to benchmark the MP produced in this study with another know similar product (i.e. already tested and legally approved MP).

As demonstrated in Figure 4.2, the crude protein content of 71% of the *Sulfuricurvum* spp. microbial culture is slightly higher than bacterial meal (68%) and fishmeal (66%) and substantially higher than the average crude protein content of soybean meal (45%).

A similar trend can be observed in Figure 4.3 for the amino acid profile. The profile for the *Sulfuricurvum* spp. microbial culture was comparable to that of bacterial meal and fishmeal and systematically better (at the exception of Arginine) than the one of soybean meal.

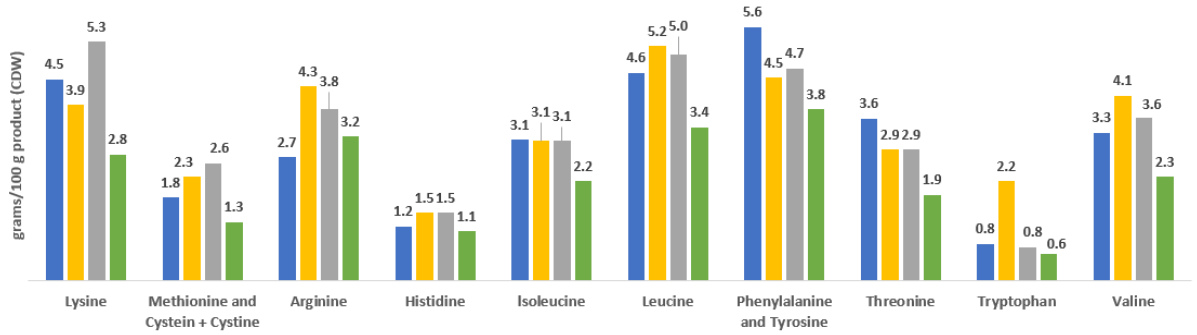

Figure 4.3. *Essential amino acids profile of the microbial biomass produced under CR configuration by the Sulfuricurvum spp. dominated culture (blue) (this study) compared with bacterial meal (yellow), fishmeal (grey) and soybean meal (green) as reported from Øverland et al.* [9].

4.4 Discussion

4.4.1 Sequencing Batch Reactor

Following the enrichment, the SBR operations confirmed that the microbial culture effectively oxidized hydrogen coupled with assimilation of carbon dioxide and mineral nitrogen (i.e. ammonium nitrogen) into cell biomass. Consistent biomass growth was observed, allowing to operate the SBR at a SRT of about 6 days. Also, the NH_4-N fed at the beginning of each SBR test was completely (100%) converted into organic nitrogen for microbial biomass build up. Nevertheless, the average performances observed in terms of volumetric productivities and biomass yield on hydrogen were far from being optimal. More specifically, the mixed culture grown under SBR conditions gave average biomass yields and productivities lower than values previously reported using specific bacterial strains (see Table 4.2).

Table 4.2. *Comparison of results obtained in this study for SBR and CR grown cultures with data from literature on single HOB strains.*

Microbial culture / Strains	Substrate	Culture method	Biomass productivity (g CDW/L·h)	Biomass yield (g CDW/g COD-H_2)	Reference
Alcaligenes eutrophus	$H_2/O_2/CO_2$	Batch	2.28	-	[67]
***Alcaligenes eutrophus* ATCC 17697^T**	$H_2/O_2/CO_2$	Batch	0.71	0.28	[63]
***Ideonella sp*. O-1**	$H_2/O_2/CO_2$	Batch	0.27	0.20	[64]
Pseudomonas hydrogenovora	$H_2/O_2/CO_2$	Batch	0.50	0.16	[164]
Mixed culture (SBR)	H_2/Air/CO_2	Batch	0.08	0.07	This study (average values)
Alcaligenes eutrophus ATCC17697	$H_2/O_2/CO_2$	Continuous	0.40	0.29	[165]
Alcaligenes hydrogenophilus	$H_2/O_2/CO_2$	Continuous	0.33	0.23	[166]
Cupriavidus eutrophus B-10646	$H_2/O_2/CO_2$	Continuous	-	0.14	[65]
***Sulfuricurvum* spp. (CR)**	H_2/Air/CO_2	Continuous	0.38	0.28	This study (average values)

The average volumetric productivity of 0.08 g CDW/L·h observed under SBR conditions, was 28.5, 9.1, 3.5, 6.4 and 3.3 times lower than the values reported for autotrophic growth of *Alcaligenes eutrophus, Alcaligenes eutrophus ATCC 17697T, Ideonella sp. O-1* and *Pseudomonas hydrogenovora*, respectively. Equally, the biomass yield on hydrogen gas was 3.8, 2.7 and 2.2 times lower than vales reported for *Alcaligenes eutrophus ATCC 17697^T, Ideonella sp. O-1* and *Pseudomonas hydrogenovora* grown under batch conditions.

The analysis of the community composition revealed a surprising fractionation of the HOB enriched community into three distinct compartments: autotrophic HOB, heterotrophic bacteria and predatory bacteria, each sharing about 1/3 of the relative abundance of the overall community. The association between primary producers

(autotrophic bacteria) and secondary consumers (heterotrophic bacteria) has already been documented in full scale MP production as well as reported and investigated in recent scientific studies [134, 145]. In the context of MP production, a clear example is represented by a methylotrophic bacterium (*Methylococcus capsulatus*) cultured in association with other heterotrophic bacteria. Such microbial fermentation is used in pilot-scale bioconversion of natural gas into MP (bacterial meal), eventually used as high-quality feed in aquaculture [145, 146]. The coexistence of different microbial species offers benefits such as the removal of inhibiting byproducts or cell lysates, as well as the regulation of oxygen level [39,134].

Quite unexpected was the 33% relative abundance of *Bdellovibrio* spp*.*, by far the most abundant genus dominating the mixed culture after 120 days of continuous SBR operations. The fact that such genus comprises predatory bacteria thriving on invasion and consumption of other Gram-negative bacteria [159] offers a reasonable yet remarkable explanation for the low performances of the HOB enriched community observed under SBR conditions. It is likely that the predatory activity of *Bdellovibrio* spp*.* imposed a major stress on the primary producers HOB, which were actively oxidizing hydrogen and fixing carbon dioxide into new microbial biomass then partly lysed by predatory activity. The lysed biomass might have also served as growth substrate for heterotrophic bacteria [167], in fact occupying the remaining 1/3 of the microbial community.

The high metabolic diversity and the low performances characterizing the microbial community under SBR condition can be also explained by speculating over the degrees of freedom of the biological systems in terms of growth rate and substrates concentrations, i.e. from a Monod-like point of view. Under SBR conditions, low constrains were imposed to the specific growth rate of the different bacteria present, which were therefore able to coexist in the same biological context. Also, the depletion of nutrients as well as the varying concentration of gasses as affected by the changing microbial activity over the batch culture (i.e. lag, log and decay phase), resulted in continuously changing growth conditions, potentially favoring different bacteria over time (see Figure 4.4a).

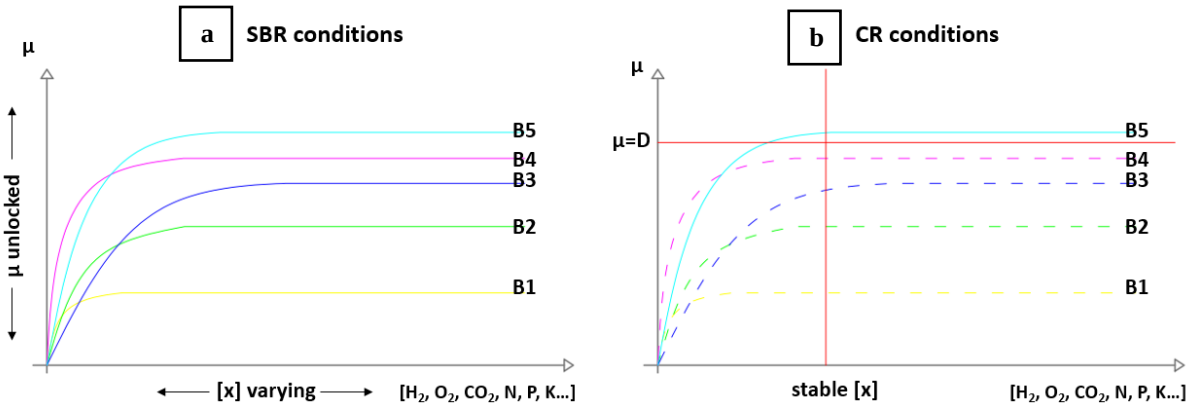

Figure 4.4. *Hypothetic resume of growth rates depending on substrates concentrations in SBR (a) and CR (b) conditions. (a) SBR culture conditions do not impose a strong growth rate to the system and substrate levels vary over the growth period, hence bacteria with different specific growth rates and affinities can freely co-evolve. (b) CR conditions are characterized by a strong dilution rate, which imposes a specific growth rate to the overall biological system. In the CR configuration substrates concentrations are well defined and become constant over time. Therefore only bacteria able to cope with the imposed dilution rate and having high affinity with the set of substrates concentrations can evolve in the system.*

In view of the sub-optimal performances, the SBR culture system did not seem to offer the best solution between process efficiency and stability. Moreover, the diverse microbial community would be difficult to control in terms of constancy of composition, and the presence of different bacterial strains of uncertain nutritional composition would affect the quality of the HOB microbiome as such for MP production for feed and food purposes.

4.4.2 Continuous Reactor

The continuous operation at a dilution rate of 0.1 h^{-1} allowed to select for the evolvement of a more performing microbial culture in terms of biomass yields and volumetric productivities. Indeed the CR culture system selected for bacteria able to implement maximum substrate conversion at the specific growth rate imposed by the dilution D at which the bioreactor is operated [7]. Thus microorganism having high specific growth rate can outcompete others not able to cope with the dynamics of the system. Such configuration can be summarized in the following two conditions:

1) $\mu \geq D=0.1\ h^{-1}$: Continuous growth

2) $\mu < D=0.1\ h^{-1}$: Wash-out

Figure 4.4b offers a virtual example of how the CR reactor impacted on the initial diverse community. The dilution rate of 0.1 h^{-1} required a corresponding specific growth rate of the same value. Moreover, the constant supply of nutrients and substrates to a biological system growing in steady conditions allowed to set a quite specific environment able to naturally select for the more adaptive and fast growing bacteria. In other terms, only bacteria possessing a specific growth rate higher than the dilution rate imposed, as well good affinities with the substrates provided were not washed-out.

As revealed by the microbial community analyses, within three weeks of operation the high dilution rate resulted in the selection of a highly specific microbial culture, dominated for more than 96% by *Sulfuricurvum* spp*.* The latter genus is known as encompassing a specific type of bacteria predominantly active towards sulfur oxidation in crude oil deposits [162]. *Sulfuricurvum kujiense* YK-1^T was first isolated from oil sands and characterized as a facultative anaerobic sulfur oxidizing bacteria (sulfide, elemental sulfur and thiosulfate) also able to use hydrogen as electron donor. Electron acceptors were described to be nitrate and oxygen under anaerobic and aerobic conditions, respectively. Aerobic growth though was limited to microaerophilic ranges (with maximum 1% in the headspace) [168]. Three other strains of *Sulfuricurvum kujiense* were already reported, but only strain YK-1T was characterized in its whole genome [162].

The *Sulfuricurvum* spp*.* dominating the culture studied in the present work is closely related to *Sulfuricurvum kujiense*, yet the exact identity of the strain is still unclear. The fact that partial pressures of O_2 in the headspace of the CR were constantly higher than 1%, reaching 5-6% for long periods, constitutes a first important difference with the strain YK-1^T as characterized by Kodama and Watanabe [168]. Although the abovementioned physiological characterization reported the use of H_2 as electron donor in combination with microaerophilic O_2 concentrations, it did not identify the possibility of exploiting such bacterium for high rate autotrophic hydrogen oxidation, as experimentally demonstrated in this study has not been described before.

As reported in Table 4.2, the cultured *Sulfuricurvum* spp*.* displayed biomass yields and volumetric productivities comparable to the ones reported for *Alcaligenes eutrophus ATCC17697* from [165], outscoring the values available in other studies for continuous cultures of *Alcaligenes hydrogenophilus* and *Cupriavidus eutrophus* B-10646 [65, 166]. The fact that the culture dominated by *Sulfuricurvum* spp*.* matched efficiencies in terms

of biomass yield and volumetric productivities of other well-known HOB strains, represents an interesting and novel finding and holds the potential to expand the biotech applications of autotrophic hydrogen oxidation to unexplored bacteria. Further research is warranted to investigate its potential in more detail.

Interestingly, the microbial composition was stable over the course of the experiments (90 days) and dominated by the same genus (see Figure 4.1). This finding is important as this implies that the fermentation process can be easily managed without cumbersome sterility precautions (e.g. media autoclaving, gas filtering). The latter feature can be of interest in allowing the direct upgrade of used resources such as carbon dioxide and ammonia gas recovered e.g. from biogas and anaerobic digestate, respectively [45], without requiring strict subsequent axenic processing conditions and related operational costs. Further research is required to understand how such operational setting is resistant to external invasion and destabilization. Indeed, the latter can have biotechnological applications which go beyond the aim of this study.

In relation to the other bacteria coexisting with the *Sulfuricurvum* spp., the spectrum was composed by heterotrophic bacteria pertaining to the classes of *Gammaproteobacteria* (*Thermomonas*) and *Flavobacteriia* (*Chryseobacterium*). It is therefore likely that under high rate CR reactor configuration, these bacteria were benefitting from organic metabolites from the HOB, in this case *Sulfuricurvum* spp. Yet, this equilibrium achieved under CR conditions was totally different from the almost equal relative abundance between HOB and heterotrophs under SBR conditions. The magnitude of the residual heterotrophic niche (in terms of relative abundance) might be indeed depending on the growth conditions, and more specifically on the dilution rate imposed to the system. Further research efforts might aim at establishing whether or not such niche would be completely diminished at higher dilution rates, not allowing the secondary heterotrophic consumers to take advantage of the primary autotrophic carbon fixation activity.

4.4.3 Nitrogen assimilation efficiency, protein and amino acids profile

In terms of nitrogen assimilation and conversion efficiency, the system operated in batch-mode was able to convert 100% of NH_4-N nitrogen into MP at 66% or more protein content on CDW basis. In case of the CR configuration, the N-usage efficiency was lower, in the order of 87% on CDW basis. The aim of the high rate CR operation

was to attain maximum biomass growth and MP accumulation, avoiding nutrient limitation. As result, nitrogen was added in a slight excess with some nitrogen was still present (unused) in the effluent of the reactor. It is likely that higher efficiencies could be obtained imposing more carefully N limiting conditions and varying the initial nitrogen loading rate.

The biomass produced under constant CR operation revealed a high protein content of more than 70%. The latter is in agreement with other studies on HOB for MP production [76], and confirms that *Sulfuricurvum* spp. might be suitable as a MP producing bacterium. The overall protein content is higher than the 68% reported for bacterial meal as well as than the one of fishmeal, regarded as high-quality additive in nutrition and also than the one of soybean meal, the reference vegetable protein for livestock. In the same way, the amino acids profile of the produced MP revealed a close compatibility to the one of bacterial meal as well as fishmeal, outscoring the one of soybean meal. Bacterial meal, as already produced from natural gas could also be used to directly upgrade the biogas produced from anaerobic digestion of sewage into MP. As discussed in a recent review [45], more than being self-excluding the hydrogen and the methane platforms can be seen as complementary, depending on the availability of each resource on-site. Like for bacterial meal, which already received positive feedback from feed trials, preliminary in vitro tests on the nutritional digestibility of our MP were also positive (data not shown). Clearly, the findings obtained in the study need to be complemented by detailed animal studies in which aspects of long-term gastro-intestinal uptake and putative nutritional side effects are scrutinized. However, the current findings clearly show the potential of using the produced MP as high-quality feed/food additive, offering a valid alternative to the high land, water, nutrients and carbon footprint of conventional vegetable protein production [143]. If this would be done by upgrading nitrogen recovered from used water the benefits in terms of avoided N losses and emissions could be even higher [13].

4.4.4 SBR and CR: general considerations

The present work allowed to compare two different culture systems: sequencing batch reactor (SBR) and continuous reactor (CR). Even though the specific conditions here tested led to conclude that the best reactor configuration for the production of MP by HOB is the continuous one, this aspect deserves more general considerations. SBR,

or more generally batch culture systems are a common practice in biotech industry for the production of different commodities from microorganisms. High volumetric productivities can be achieved under SBR configuration, and more importantly elevated final concentrations of product at the end of the batch enable better downstream processing of the final product. On the other hand, especially when dealing with gas fermentation techniques, gas transfer limitations might occur once concentration of biomass or other metabolites becomes too high, therefore counteracting the advantage obtained in the downstream processing step.

CR configuration systems, if allowing constant and stable culture conditions, require more accurate process control and often cannot achieve the final concentrations of product measured in batch systems.

In the research work here presented, the SBR was operated under non-axenic conditions and with a mixed microbial community, whereas stability of biotech processes employing SBR and axenic cultures usually requires accurate sterilization of the reactor as well as of the components fed to the biological system. It might be speculated that if the *Bdellovibrio* spp. was not present in the enriched HOB microbiome, the performances of the SBR system could have been comparable to the CR in terms of process stability and biomass productivity, having the additional advantage of achieving higher final biomass concentrations. Nevertheless, it is apparent that under such reactor system the microbial community will vary in composition over time. In fact, the microbial community composition observed at the end of the enrichment step (see Figure 3.5), evolved to a completely different one after the 120 days of SBR operations (see Figure 4.1). On the other hand, the high selection process operated under CR reactor conditions allowed to have a much more stable community composition over more than 3 months of continuous operations. To complete the comparison amongst the two systems under the specific conditions of the present work, it would be interesting to see how the highly stable *Sulfuricurvum* spp. dominated culture obtained under CR conditions, would evolve under SBR configuration. In case process stability and efficiency would be confirmed by a constant composition of the culture also under SBR conditions operated without sterility measures, it might be concluded that SBR would then be a better configuration than CR, still not requiring axenic measures but allowing higher final product concentrations, therefore enabling better downstream processing of the produced MP.

4.5 Conclusions

In this study, we aimed at assessing the potentialities of autotrophic hydrogen oxidation to recover and upgrade of resources under different operating conditions. The evolution of HOB from a generic mixed microbial community under different operating conditions allowed to reveal interesting and novel aspects, with potential for application in industrial contexts. The key findings are:

- Under SBR conditions the enriched mixed culture revealed the coexistence of a diversity of microbial actuators
- Under high rate CR culture conditions the microbiome narrowed down to *Sulfuricurvum* spp. dominated culture which was both stable and highly productive
- Mineral nitrogen and carbon dioxide were directly upgraded into microbial biomass, rich in protein, by using hydrogen and oxygen with high efficiency under CR culture conditions;
- The nutritional properties of the produced MP are comparable to the high-quality fishmeal and surpass those of vegetable soybean meal.

Microbial biosynthesis of useful commodities from carbon dioxide is amongst the most challenging yet promising routes of the future bioeconomy. The exploration of renewable energy generation combined with technology advances in hydrogen production might enable on-site recovery and upgrading of valuable resources by means of HOB, produced under appropriate microbial resource management (MRM) conditions [144, 169].

4.6 Acknowledgments

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CHAPTER

5

CAN DIRECT CONVERSION OF USED NITROGEN TO NEW FEED AND PROTEIN HELP FEED THE WORLD?

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CHAPTER 5

CAN DIRECT CONVERSION OF USED NITROGEN TO NEW FEED AND PROTEIN HELP FEED THE WORLD?

Abstract

The increase in the world population, vulnerability of conventional crop production to climate change, and population shifts to megacities justify a re-examination of current methods of converting reactive nitrogen to dinitrogen gas in sewage and waste treatment plants. Indeed, by up-grading treatment plants to factories in which the incoming materials are first deconstructed to units such as ammonia, carbon dioxide and clean minerals, one can implement a highly intensive and efficient microbial re-synthesis process in which the used nitrogen is harvested as microbial protein (at efficiencies close to 100%). This can be used for animal feed and food purposes. The technology for recovery of reactive nitrogen as microbial protein is available but a change of mindset needs to be achieved to make such recovery acceptable.

5.1 Introduction

Sustainable boundaries of human activities on earth have been widely identified and are of strong concern. Top of the list are ecological diversity, climate change, the terrestrial water balance, and the impact of nitrogen on the overall ecosystem [170]. There are clear links between different boundaries. Indeed, climate change is directly linked with CO_2 emissions, but the latter has a strong connection with the anthropogenic nitrogen impact (i.e., fertilizer) used to produce feed and food. About 1-2% of the total world energy consumption is used to produce reactive nitrogen by means of the Haber Bosch process. Current anthropogenic sources of nitrogen are 100 Mt of nitrogen by chemical fixation, 35 Mt by biological crop fixation and 10 Mt by atmospheric deposition in animal rearing. Yet of this total only 13 Mt nitrogen are consumed as vegetable protein and 10 Mt nitrogen as animal protein, totalling only a mere 16% net efficiency. These massive losses in the nitrogen cycle are largely due to losses during primary (plant) agriculture (runoff, leaching, volatilization and denitrification). Losses are high because plant agriculture is the entry point for nitrogen to the food chain (and hence the largest amount is at this point). In addition, nitrogen entering waste streams is currently mainly converted to dinitrogen gas and lost to the atmosphere rather than re-used to make food. Indeed, wastes generated by animals and humans not only generate greenhouse gases (N_2O and CH_4) but also destroy resources which could, by proper recycling, help to abate climate change. In this work, problems relating to the energy demanding production of reactive nitrogen by industry or by recovery processes from wastes, and the overall ineffective use of nitrogen in the conventional agro-system are examined. Subsequently, a new approach is proposed in which the used nitrogen is converted to single cell microbial protein to be used as feed and food. Finally, the overall impact of such direct conversion for the planet, in the context of population urbanization towards megacities, is evaluated.

5.2 Better nitrogen management is pivotal for a sustainable feed/food supply

Nitrogen (N) in its reactive forms (ammonium, nitrite and nitrate) is essential for plant growth and thus for synthesis of proteins to be supplied to animals and humans. While N constitutes almost 80% of the terrestrial atmosphere, its availability in a reactive form

is limited. The supply of biologically available nitrogen relying on biofixation (leguminous crops), atmospheric deposition, or on crop residues, fecal matter and animal manure recycling covers only about half of the present agricultural demand, largely due to enhancement in agricultural plant growth rates through supply of chemically derived reduced nitrogen [171]. Indeed, since the Haber-Bosch process was invented in the early 1900, industrial production of N-based fertilizers and better seeds supported the largest historical increase in food production capacity [172]. As direct consequence, the global population has reached levels which otherwise could not be achieved. Further growth is expected to bring world population between 8 and 10 billion by 2050 [3], resulting in substantial pressure on food supply, especially in terms of high value protein supply. This supply of industrial fertilizer changed the nitrogen cycle, with 30% of terrestrial nitrogen being generated from human activities (mainly due to fertilizer production and utilization) [173], and this is projected to rise [174].

At present, of the nitrogen used as fertilizer, only a few percent is effectively consumed as food protein, particularly if the majority is consumed as meat protein. Fertilization by industrial N-fertilizer suffers from a number of inherent losses. Overall, losses for runoff, leaching, ammonia volatilization and denitrification make up from 50 to 70 % of the initial amount of N supplied as fertilizer [175]. Different solutions have been proposed to solve the high inefficiencies in agriculture. Methods such as genetic modification of non-leguminous crops to achieve biological nitrogen fixation, application of slow-release fertilizers or coated fertilizer in order to decrease run-off [176] have been proposed. Yet, none of them has been able so far to solve such problem at large scale, and massive industrial nitrogen fertilizer inputs are still needed to support high agricultural production of feed and food.

The other key inefficiency is generation of meat protein from plant protein, with unconverted nitrogen being discharged as manure, of which only 50% is reused as fertilizer.[177] The feed-meat nitrogen conversion ratio depends heavily on species, overall feed conversion ratios (FCR – kg dry feed per kg whole animal weight gain), ranges from 1.5-2 for chickens, to 3 for pigs, to 7-20 for sheep and cattle [178], with intensive livestock generally having advantageous ratios. As dry grain feeds and whole animal nitrogen are both on the order of 2%-3% N (mass basis), FCR is approximately reciprocal to nitrogen conversion efficiency (kgN_{fed}/kgN_{animal}). The current land area devoted to livestock feed and production constitutes about 75-80% of the total

agricultural land use [179]. This makes the industrial N-fertilizer production and its massive utilization for protein supply a serious concern in terms of its large environmental footprint. Actually, nitrogen manufacturing exceeds the estimated sustainable boundaries by a factor of 5 [173, 178, 180]. It has been calculated that the industrial production of N-based fertilizers by the Haber-Bosch process constitutes about 1-2% of the world power generation,[181] with 4 to 8 tons of CO_2-eqv per ton N fertilizer produced [182]. Ammonia and nitrate loss to the environment causes eutrophication, and nitrification contributes to agricultural and environmental N_2O emissions, a gas with a greenhouse warming potential 300 times higher (on a mass basis) than CO_2, and with the highest impact on ozone depletion amongst other ozone-depleting gasses [183, 184]. Because of the major role played by nitrification, N_2O emissions from agriculture account for about 25% of the global N_2O emissions[185].

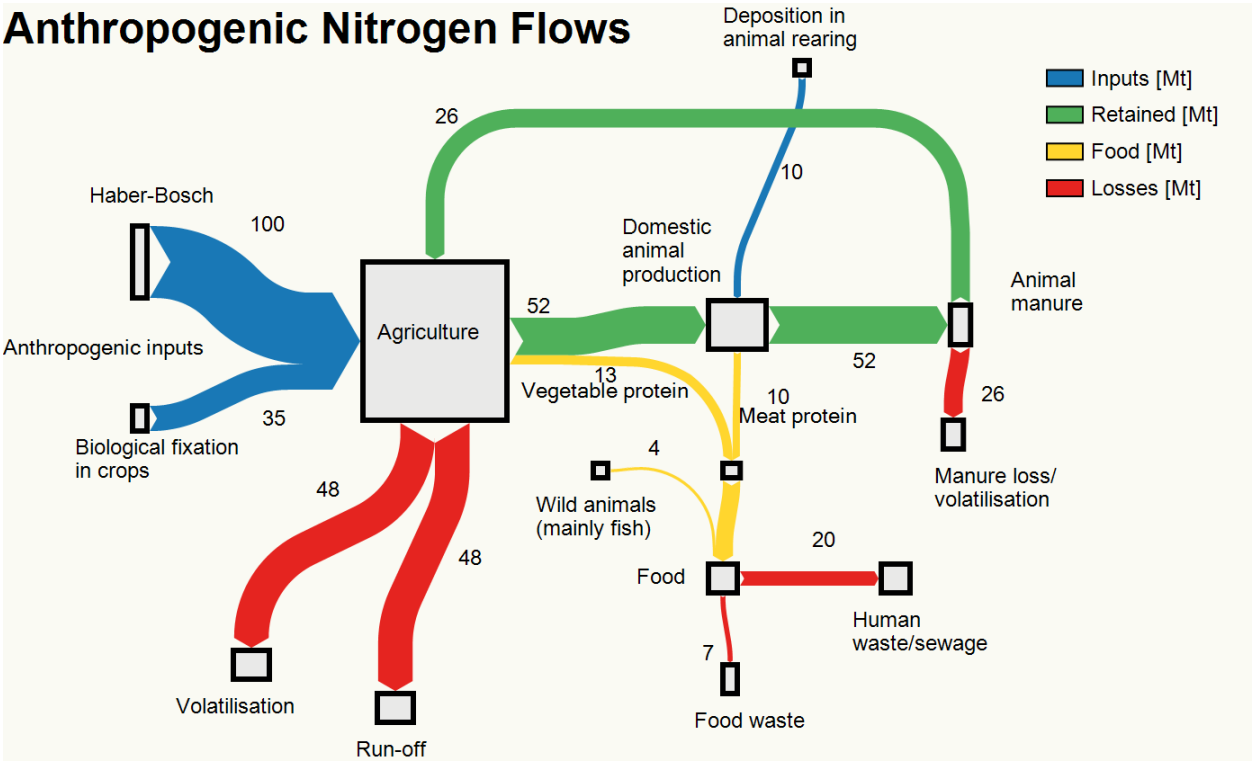

Figure 5.1. *Anthropogenic nitrogen cycle proportional to current Haber-Bosch fixation (100 Mt), with a focus on industrialised agriculture. Calculated based on agricultural N-utilization efficiency of 40%* [175]*, feed conversion efficiency of 15%* [178]*, manure utilization of 50%* [177]*, and with proportional nitrogen input fluxes taken from* [173] *and* [186]*. Of 135 million tons N entering the agricultural process (Haber-Bosch, Biological fixation in crops), 17% is retained in vegetable and meat protein, and 15% in the urban wastewater process. The remainder is dissipated to the natural environment. See also Bodirsky* [176, 187] *which was done independently in global simulation software, but which matches these calculations.*

If feed/food supply continues to rely on the present soil-plant based production system, the increasing demand for edible protein in the near future will exacerbate even more these aspects [183]. Already at present, about 30% of all ice-free land, 70% of freshwater and 20% of energy are used in the feed and food production system [179]. Global fertilizer consumption is expected to increase by 50% by 2050 to sustain the increase needed in food production capacity [174]. Therefore, a more sustainable and efficient route for nitrogen conversion into edible protein needs to be found, especially when considering the inherent losses in the nitrogen cycle, with a large fraction dissipated in both plant and animal production (Figure 5.1). A range of alternative options have been proposed to improve nitrogen usage, including a change in diet to reduce animal protein consumption, improved efficiencies in fertilizer application and animal rearing, and better resource management [176]. As an example, switching world diets to only vegetarian would have a dramatic impact, but a more realistic scenario of limiting animal protein intake to no more than 29% of total protein intake, has nitrogen usage efficiency gain of about 20% only [176]. The key issue is that none of these options fully address the major losses involved with open-field plant agriculture. Here we propose an alternative pathway, which is up-cycling of used nitrogen directly to microbial protein, which would enable productivity gains independent of agricultural production.

5.3 Role of climate change in the need for new feed and food

Climate change is another factor affecting the goal of feeding the world. First of all, increases in soil temperature might accelerate microbial conversion of organic matter and nitrogen, thus enhancing the losses of both in the soil ecosystem [188]. Moreover, if the climate decreases our ability to produce food, particularly in sub-Saharan Africa, south Asia, and Latin America our prospects for feeding the world become dismal. The Intergovernmental Panel on Climate Change estimates that climate could reduce global crops production yield by 10% by 2050, with regional variations reaching up to -50% [189]. Production of protein using direct conversion of mineral nitrogen to microbial protein is less climate sensitive and can help alleviate these stresses. In recent years, our global average caloric intake rose to a respectable 11.6 MJ/person·day based on new protein sources [190]. Country-by-country and commodity-by-commodity projections indicate that this quantity could rise to 12.8

MJ/person·day by 2050. A growth in agricultural production of at least 60% would be needed, at the same time that population is projected to increase by 37% during the period 2005−2050. A relevant fraction of that 60% requirement could be reduced by reuse of nitrogen in wastewater by microbial growth.

5.4 Using energy to dissipate nitrogen: sewage treatment of urban wastes

Taking urban wastewater as the key example, protein consumed as food is excreted mainly as urea and NH_4^+ by human metabolism, and discharged to the sewer. The amount of N excreted as a fraction of that fixed by the Haber Bosch process varies from 18%-30%, with lower levels in industrialised nations due to losses in animal conversion (21% in our example) (see Figure 5.1) [191]. Current sewage treatment technology is based on the Conventional Activated Sludge (CAS) process, which dissipates nitrogen through the nitrification/denitrification or deammonification process [103]. Reduced, reactive nitrogen is hence biologically converted to its nonreactive dinitrogen gas form, and then released back into the atmosphere, with N_2O gas emissions representing an intermediate of increasing concern in terms of greenhouse gas (GHG) emission from WWTP [192]. In terms of energy consumption, the two processes of N-fixation for fertilizers production and N-dissipation for wastewater treatment are comparable, both requiring around 40 MJ/kg N fixed or dissipated [191]. Therefore the present sewage treatment system destroys reactive nitrogen using the same amount of energy as used to fix it into fertilizers. While there has been a strong focus on retrieving the direct energy value of the organics present in used waters (used mainly to produce biogas), recovering mineral nitrogen potentially represents an equivalent gain, with broad applicability beyond urban contexts to agro-industrial streams for direct nitrogen recovery to avoid its dissipation in the environment.

5.5 Cost-effective nitrogen recovery: can this be achieved?

It is generally not considered justified to produce fertilizers from nitrogen recovered from fecal matter, urine, sewage, etc. at higher energy expenditure than is needed by the Haber-Bosch process [193]. However, this viewpoint does not consider non-scope 1 carbon emissions (i.e., those relating to transport, downstream processing etc.), or

emissions relating to fertilizer formulation and distribution, and wastewater treatment of produced nitrogen, which are highly region specific. This can make the recovery of nitrogen from various streams, particularly where there is no existing wastewater treatment infrastructure or wholesale replacement is required, a more appealing approach. Physico-chemical and biological processes, as well as the combination of both have been studied and implemented for nitrogen removal and recovery from used water. A comparison between the energy requirements of biological, physico-chemical and combined N-removal/recovery techniques is shown in Table 5.1. As shown most of the “best” established recovery technologies such as struvite precipitation, adsorption, electrodialysis and air stripping are still not competitive with the Haber-Bosch manufacturing, though they avoid the costs of ammonia manufacturing. Indeed, all these techniques are usually applied at relative small scale and although they recover the nitrogen, they are more expensive than the Haber-Bosch process, which is practiced at such massive industrial level that it profits from the dimensions of scale.

Table 5.1. *Different scenarios for N routes. Primary energy requirement are compared for reactive nitrogen production by conventional industrial processes, reactive nitrogen removal and recovery from used water as well as reactive nitrogen recycle by the combination of different routes. A conversion efficiency of 0.31 was used to convert the electricity consumption to primary energy. Source:* [193].

Scenario	Route	Process No.		Energy requirement MJ/kg N	Potential use [d]	Ref.
N-production: N_2 to NH_3	Fixation	1	Ammonia production (best available technology)	37	N.A.	[193]
		2	Average N-fertilizer production Europe	45	N.A.	[193]
		3	Average ammonia production Europe	43	N.A.	[193]
N-removal: NH_3 to N_2	Biological	4	Nitrification/pre-denitrification in WWTP (CAS)	45	N.A.	[193]
		5	Mainstream deammonification	12	N.A.	[194]
N-recycle	Physico-chemical	6	Thermal volume reduction of stabilized urine [a]	34	CP	[193]
		7	Volume reduction of stabilized urine with reverse osmosis [b]	29	CP	[193]
		8	Struvite precipitation for P recovery	69	CP, FF	[195]
		9	Adsorption (ion exchange)	116	CP, IA, FF	[196]
		10	Electrodialysis	65	CP, IA, FF	[197]
		11	Stripping with air and $(NH_4)_2SO_4$ production	90	CP, IA, FF	[193]
	Combinations	12	Anammox + Haber-Bosch	54	N.A.	[198]
		13	Bio Electrochemical System (BES) [c]	-11	N.A.	[199]

[a] *Calculated for urine: 10 fold concentration with vapour compression*

[b] *Calculated for urine: 5 fold concentration*

[c] *Calculated for a microbial fuel cell (MEC) treating urine and recovering nitrogen via air stripping; as yet only at lab-scale*

[d] *Potential uses of the physico-chemical recycled N: process 6 to 11. CP=Crop Production, IA=Industrial Application (such as DeNOx, synthesis of N-polymers), FF=Feed and Food, N.A.= Not Applicable*

Of particular interest are the combined physico-chemical and biological approaches for the removal of used N and production of a new usable form. In this context, process no. 12 demonstrates how a biological N-removal technique (Anammox) succeeded by the Haber-Bosch provides an efficient way of handling nitrogen, although this represents only a theoretical concept with the atmosphere as overall N-pool. The process no. 13 utilizes the inherent chemical energy in urine to drive electro-pervaporative ammonia recovery. However, it relies on source separation to urine, has only been reported in lab scale [129, 199, 200]. Improvements of the stripping efficiency and the implementation of further technical advances already proposed for

other electrochemical systems treating N-rich streams will make these innovative systems more practical [113].

Overall, the current mature recovery technologies can achieve energy parity with Haber-Bosch manufacturing, only on concentrated wastewaters such as from certain food industries. This does not consider economic issues, and the cost of these technologies (particularly bio-electrochemical, or pervaporative systems given the high capex costs) drives the cost per unit nitrogen above the current market price of $700/ton [201]. As an alternative to nitrogen recovery and reuse, the long path of dinitrogen to plant or animal edible protein-N should be critically examined; there is a need for a shorter more effective route.

5.6 Conventional vs direct reuse: up-cycling nitrogen by a short route

At this moment, the anthropogenic and natural nitrogen cycles interact and pool to create vegetable and animal proteins [202], with by far the largest magnitude of loss occurring through production of plant protein, mainly because the largest amount of nitrogen enters plant agriculture and hence absolute losses are high. Waste derived nitrogen both from human and animal can be reused directly as a field fertilizer, either as a concentrate (recovered using technologies identified in the previous section), or directly reused following primary treatment and a form of hygienization [203]. This is of course, widely applied to animal manures, and 50% of all animal manure is recycled for agricultural purposes [177]. This latter case saves 40 MJ/kg sewage-N, but this has limited applicability, due to seasonal variations, cost of transport particularly in case of sewage N (limited also by presence of heavy metals and micropollutants), and limits in agricultural land near urban centers. Moreover, once applied in the field, the sewage ammoniacal N will be subject to large field losses for instance by run-off of soluble NH_4^+ and NO_3^- and by volatilization of NH_3, N_2O and N_2.

An alternative, which inherently avoids losses in field production from plants, is direct production of animal-edible proteins from used nitrogen. This means that the nitrogen cycle is short-circuited in the most direct way, avoiding all the inherent losses of crops or even potentially livestock production.

5.7 Recovered nitrogen: nothing new

Recovered nitrogen is indeed already part of our daily life. Production of edible mushrooms is actually based on the direct use of organic wastes such as agricultural by-products (particularly the N-rich chicken manure) as well as industrial and municipal wastes [204]. In this way, used nitrogen is directly incorporated into valuable and edible fungal matter. Another practice of direct up-cycling of fecal nitrogen to edible protein is currently ongoing in aquaculture. The nitrogen excreted by fish, instead of being treated and neutralized by means of the traditional biological nitrification/denitrification, is incorporated by the so-called biofloc technology in new microbial biomass rich in protein [109, 110]. This has enabled a dramatic shift in sustainability and feed-conversion levels in aquaculture [205] for generating low-cost fish protein that can also be realized for other species.

5.8 Biotechnologies for direct upcycling of used N

Bacteria or microalgae can be used directly in the assimilative partitioning of reactive nitrogen supplied or recovered from wastewater [206, 207]. Particularly fast growing photosynthetic microalgae and bacteria, and, phototrophic and organotrophic bacteria can be used to completely exhaust the reactive nitrogen by taking it up in cell biomass and form animal digestible protein.[6, 208] In this case, the outcome is a high uptake of the nitrogen in N-rich biomass [209] which can be used, for example, as fertilizer [206, 207] but also as feed or food.

Microbes have been extensively and historically studied as potential producers of feed and food, and their actual use is visible in our daily life [6, 81]. Yeast, for example, represents a direct microbial source of food or food additive.

Chemotrophs are interesting as they allow recovery of carbon dioxide present in the water or in biogas and energy, as well as nitrogen from wastewater. Methylotrophs, which are bacteria able to grow on natural gas, have been studied extensively as possible source of single cell protein [92], i.e. protein accumulated by single cell microorganisms [6]. They are already used, for example, as feed for aquaculture.[142] Organotrophic single cell protein production is possible as feed and food protein producer [92, 211]. In this case an inexpensive and available organic carbon present in the form of residual organics derived from the original vegetable matter (e.g. food

industry process water) is used to grow microbial biomass able to accumulate proteins. Other kinds of bacteria suitable as potential protein producers are lithotrophic bacteria, using molecular hydrogen to fix carbon dioxide into protein-rich biomass [45]. All the above mentioned microbial species might be useful to up-cycle used reactive nitrogen directly to edible protein. The question arises to what extent this route of biotechnological direct conversion of used N can represent a valid alternative to the established anthropogenic N cycle. It is generally accepted that about 200 to 500 MJ of electron donor (organic carbon, hydrogen or methane) are required for the production of 1 kg of microbial N (see Table 5.2). In case the latter is harvested and consumed directly, it appears that direct nitrogen conversion is highly advantageous and clearly offers perspectives for up-cycling fecal nitrogen, even via the route of stripping and upgrading the stripped N in the form of single cell protein (Table 5.2). In all fairness, the issues related to the quality of the edible protein (plant-animal-single cell protein) are not integrated in this discussion, but certainly are of value.

Photosynthetic organisms reduce inorganic carbon (carbon-dioxide) by deriving electrons from water to produce oxygen to generate organics. They are generally inefficient in terms of light efficiency (<9%) but they can utilize natural light [212]. They hence require large amounts of space to drive substantial nitrogen uptake. Microalgae are an effective source of protein but with lower digestibility than bacteria [213]. Electrical consumption in operating large-scale photobioreactors can also be substantial, due to requirements for microalgae harvesting and CO_2 delivery, and can be on the same order as the energy harvested through photosynthesis [214]. Phototrophic organisms such as purple phototrophic bacteria are an interesting alternative, as they utilize infra-red light to drive organotrophic uptake of soluble organics (similarly to how organotrophs utilize chemical energy to drive growth), without producing oxygen, including on domestic wastewater [206]. Only small amounts of light energy are needed to drive organic uptake because growth is anoxygenic (i.e., phototrophic not photosynthetic). Phototrophic bacteria are effective as animal feed [215].

The key restriction for microbial food/feed production from wastewater is potential contamination of the product for instance with pathogens. Only in specific cases such as food processing wastewater is there a case for direct-contact assimilation from wastewater [211, 216, 217]. Microorganisms might, on the other hand, serve as a vehicle for the direct assimilation of nitrogen recovered as mentioned in Table 5.1. In

this case the produced biomass will be of hygienic quality, and the absence of direct contact with wastewater allows their use as animal feedstuff or even as human food. This might be achieved, for instance, by coupling an N-recovery technique with the intensive production of such protein rich microbial biomass. In this sense anaerobic digestion would play a key role, converting most of the organic N embedded in the wastewater into ammonium. The latter could be then recovered from concentrated streams (e.g. digestate) by means of physico-chemical processes such as stripping, membrane technology, adsorption etc. After further polishing, the liquid and/or gaseous recovered ammonium stream could be finally integrated in microbial cells to be harvested as feed or food.

Table 5.2. *Energy needed to produce 1kg edible protein nitrogen with conventional route (meat protein) and through microbial growth (single cell protein)*

Protein production system		Energy source	Carbon conversion efficiency	MJ/kg N-protein [a]	Advantages (+) and Disadvantages (-)
Conventional route: **edible meat protein**		Fossil fuel	n.a.	4000 [218] [b]	**+** *Consolidated technology* **-** *High inefficiencies, environmental burdens and land requirement*
Microbial protein	Organotrophic	Organic matter [c]	Organic-C to cell-C: 0.3-0.4	230 [219, 220]	*+ Applicable in a circular bio-based economy; sustainable; minimal land requirement* *- Energy requirement for processing the biomass*
	Lithotrophic	Hydrogen gas	CO_2-C to cell-C: 1.0	452 [221]	
	Methylotrophic	Methane gas	CH_4-C to cell-C: 0.1-0.2	361 [219, 220]	
	Phototrophic (anaerobic phototrophic bacteria)	Organic matter, Light	Organic-C to cell-C: 1.0	450 [d] [206, 215]	
	Photosynthetic (microalgae)	Light	CO_2-C to cell-C: 1.0	5000 [e] [213]	*+ Natural light only* *- High footprint*

[a] *For the single cell protein production, a biomass composition of $C_1H_{1.8}O_{0.5}N_{0.2}$ was assumed as reference for the N-content*

[b] *Beef cattle was used as reference for meat protein production*

[c] *Acetate was considered as organic carbon substrate. A value of 2MJ/kg O_2 was considered for aeration.*

[d] *Phototrophic bacteria: 80% of energy delivered chemically, 20% as infrared light.*

[e] *Photosynthesis light as chemical energy.*

These approaches engage with the emerging biorefineries concept, in which mixed, low value organic material is fed to a multi-route and converted to value added products [222, 223]. For instance, nitrogen can be processed to generate organo-nitrogen chemicals, including amino acids, through catalytic conversion. These can be used

directly as feeds, or even to generate very high nitrogen fertilizers such as citrulline, which are more effective and less readily lost to volatilization compared with chemical nitrogen [224].

5.9 Future megacities and mega N-fluxes

Recovery of nitrogen from urban centers requires a major reimagining of wastewater treatment, as the current paradigm of conventional activated sludge only allows for recovery of 20% of the N which accumulates in waste sludge [101]. In view of enhancing the recovery of used nitrogen and improving its economic feasibility, new concepts for wastewater treatment should be applied within the urban water cycle [49, 51–53]. Pre-concentrating organic carbon and nutrients at the head of the main treatment line for instance by means of High Rate Activated Sludge (HRAS) will allow, for example, to recover maximum energy (in form of biogas) and resources (nutrients and minerals) after digesting the concentrated stream of the “minor water line”, as well as water reuse on the “major water line” [225]. This so-called Major&Minor (M&M) water line approach shifts the focus from dissipation to resource reclamation. The amount of reactive nitrogen discharged in the sewer is only a small fraction (10-30%) compared to what is used in agriculture for crops and livestock production [191]. This emphasizes once again the importance to explore the microbial cell protein recovery route (Table 5.2). It is also important to identify which of the technology methods as mentioned in Table 5.2 for direct recovery of nitrogen can be applied to animal manures, and hence make meat protein production more efficient while offering unchanged consumer products. The technology for this is already appearing in the market, and directly utilizes the natural advantages of concentrated and degradable animal manure [226].

Besides the substantial global population growth expected in the near future, another relevant phenomenon will contribute to affect the nitrogen cycle. The concentration of people in metropolitan areas has been already recognized as one of the trends that will re-shape our lives [227]. The (over)growth of the so-called megacities will pose several issues in terms of sustainability [228, 229]. Feeding millions of people living in focused urban locations will be one of the main challenges. The expansion of the cities will subtract arable land from agriculture, which is already suffering from a lack of land availability, and suggests the implementation of urban farming where possible. At the

same time, massive amounts of nitrogen in the form of food protein will be supplied to these urban areas, and will leave via the sewage system. Treating such large quantities of reactive nitrogen will be needed to avoid its regional impact [230]. The fact that microbial nitrogen recovery can be designed so that it occurs in intensive reactor systems with a small footprint is in this context of significant importance. Indeed, if one assumes the production of single cell protein based on hydrogen (produced from renewable electricity by electrolysis of water or reforming of biogas) [117, 231], carbon dioxide (e.g. from biogas) and recovered ammonia, the production of the latter in high density reactor systems can surpass the normal plant protein by several orders of magnitude in terms of physical footprint. As example, in case of water electrolysis powered by photovoltaic energy (average solar radiance of 1800 kWh/m^2·year, photovoltaic conversion efficiency of 15%, electrolysis efficiency of 82%) an unit of land would deliver an equivalent of 5.6 kg H_2/m^2·year (39.4 kWh/kg H_2), corresponding to actual 2.8 kg H_2/m^2·year (1 m^2 of photovoltaic panels requires 2 m^2 of available land).[232, 233] Wind energy, at an average power per unit area of 2W/m^2 would deliver about 1300 kg H_2/m^2·year [234]. Using the latter as energetic substrate to support the growth of single cell protein (hydrogen-oxidizing bacteria with a yield of 2.4 kg dry biomass/kg H_2) [45], could yield up to 67 and 3120 tons/hectare·year of microbial protein respectively for solar and wind based systems. Compared to current soy productivity of about 3 tons/hectare·year [235], microbial protein production by means of renewable hydrogen is potentially 1 to 3 orders of magnitude more efficient in terms of land use.

5.10 Up-cycling used nitrogen to feed the future

Up-cycling used reactive nitrogen directly to feed or food can be seen as a possible way to reduce the dependency of food supply from conventional agriculture. The use of phototrophic, organotrophic or lithotrophic bacteria must be considered as crucial processes to feed the increasing future global population. Exploiting sun light or inexpensive organic carbon substrates, respectively for phototrophic and organotrophic bacteria, to assimilate and up-cycle recovered nitrogen might open new promising perspectives. Lithotrophic hydrogenotrophic bacteria could be utilized with off-peak green energy and ammonium recovery to produce directly high value protein at low energy costs and net environmental benefits (CO_2 capture). The inherent fear

related to the use of microorganisms for food must be overcome by education as well as application of safe and effective technology. Managing the anthropogenic nitrogen cycle in a more efficient way will be therefore of crucial importance for meeting the future global food challenge in a new and sustainable way.

5.11 Acknowledgments

We thank Prof. Damien Batstone, Dr. Tim Huelsen and Prof. Jerald Schnoor for their meaningful collaboration to the work reported in the present chapter.

Chapter 6

Anaerobic digestion as a platform for nitrogen upcycle by means of hydrogen-oxidizing bacteria: a case study

CHAPTER 6

ANAEROBIC DIGESTION AS A PLATFORM FOR NITROGEN UPCYCLE BY MEANS OF HYDROGEN-OXIDIZING BACTERIA: A CASE STUDY

Abstract

Anaerobic digestion plays a central role in the context of resource recovery from used water. Concentrating organic carbon and nutrients (N, P) up-front, the treatment facilities can be effective for generating a concentrated stream to be digested anaerobically. Both physico-chemical and biological processes are suitable for this concentration step. Anaerobic digestion can subsequently bring forward maximum organic carbon recovery in the form of biogas and moreover generate a digestate fit for nutrient recovery. Besides the conventional nutrient recovery techniques (N and P to become plant fertilizers), nutrient management can be improved by generating valuable edible microbial protein.

6.1 Introduction

Water and food supply are being increasingly challenged by global climate change and population growth. Concomitantly, anthropogenic exploitation of natural resources boosts the climate change, thereby causing lower productivity and depletion of natural resources. Amongst other established processes, also the treatment of used water must be revised in order to interrupt this unsustainable approach. A shift from a dissipative technology to a re-generative one can be key to revert the role of used water. At present used water is a waste to be neutralized (with net energy consumption); in the future it will be a multi-resource to be regenerated and upcycled (with an overall environmental benefit).

Nitrogen, conventionally transformed from its reactive forms (ammonium, nitrite and nitrate) to the non-reactive dinitrogen gas by the nitrification/denitrification process, can also be recovered and upcycled as main constituent of new useful products. A substantial fraction of the mineral and organic components in the influent can be subjected to anaerobic digestion after being processed and concentrated by means of high-rate activated sludge. The rest can be recovered by ultrafiltration/reverse osmosis (UF/RO) [225]. Different physico-chemical techniques such as dynamic sand filtration, chemically enhanced primary sedimentation and poly-electrolyte addition are available for pre-concentration of the COD at the entrance of the waste treatment plant. The most interesting biological alternative is represented by the use of bio-sorptive High Rate Activated Sludge (HRAS), offering the advantage of adsorbing both particulate COD and organic nitrogen. Within this configuration, dissolved nitrogen is only partly used by the microbial biomass in the assimilatory metabolism, with the remaining fraction to be treated by conventional destructive deammonification processes in the main treatment line [49]. A more technologically complex alternative is the implementation of the above mentioned advanced treatments such as UF/RO. The latter are more expensive than deammonification; yet they allow final water reuse (up to potable standards) and ammonium nitrogen captation in concentrates (brines). The total ammonia released from digestate and the part collected in the filtration brines can subsequently be used for up-cycling. The recovery of such concentrated nutrients (N and P) from digestate by means of conventional physico-chemical techniques is usually aimed at the production of plant fertilizers such as ammonium sulphate or struvite. Unfortunately, the latter products are of low value, and if used for the

production of crops to be used as livestock feed, they are lost in the soil-crop system for more than 60% (volatilization, leaching and runoff) [175]. A better and more efficient recovery strategy for nutrients, and especially for reactive nitrogen, is represented by the direct upcycling of the nutrients to edible microbial protein.

Microbial re-assimilation of recovered nitrogen, driven by renewable electron donors for the microbes already present or producible on-site (i.e. the water treatment plant), can contribute to the extra-step for the regeneration of recovered nitrogen into edible microbial protein. The latter process can be carried out at efficiencies close to 100% in terms of nitrogen assimilated into new proteinaceous biomass. Heterotrophic, lithotrophic, methylotrophic and phototrophic microorganisms are the main metabolic routes which can lead to the re-assimilation of nitrogen into new biomass, which can be harvested and valorised as high protein feed additive [13]. This approach offers the advantage of avoiding the losses which normally occur in the conventional agro-practice of chemical fertilizer utilization.

Methanotrophic metabolism can be also of value for this purpose, and of course the availability of biogas on site is one of the main advantages of this kind of bacteria. Nevertheless, this methane-based process faces several issues such as low yields, low growth rates and self-inhibition, which might hamper its implementation as microbial process for efficient N re-assimilation and upcycling [236].

In the ideal scenario here depicted, the lithotrophic hydrogen oxidation and inorganic carbon (CO_2) fixation is the metabolic pathway considered for N re-assimilation. In this sense, hydrogen-oxidizing bacteria can in fact integrate nutrients (N but also P) and bring about energy recovery in a final desirable product such as high-value microbial protein to be used as feed or food supplement. These robust and versatile bacteria, possessing the ability to oxidize hydrogen in presence of low levels of oxygen, have the capability to fix carbon dioxide and build-up high-value bacterial protein. In our specific perspective, the protein produced is based on recovered reactive nitrogen. Substrates for microbial nitrogen re-assimilation and upcycling as microbial protein can be found, on-site, in the hydrogen produced for example by water electrolysis powered by renewable energies (wind, solar etc.) [45], or by the reforming of biogas in the so-called tri-generation process [152, 153]. The latter combines heat, hydrogen and power (CHHP) generation. Besides heat and energy, the CHHP process generates also hydrogen from biogas reforming, which can be used as substrate for hydrogen-

oxidizing bacteria. A case study for on-site regeneration of recovered reactive nitrogen by means of hydrogen oxidizing bacteria, driven by anaerobic digestion as central platform, is presented subsequently.

6.2 The case study: 100 000 inhabitant equivalents used water factory

The feasibility of on-site nitrogen regeneration and upcycling by hydrogen-oxidizing bacteria has been analysed in the context of a treatment plant of 100 000 inhabitant equivalents (i.e.). In this case, an average input in of 250 L/i.e.·d can be assumed, for a total influent of 25 000 m^3/d of used water. The average pro capita input of 13 g N/i.e.·d [225] accounts for a total influent nitrogen in the treatment plant of 1300 kg per day. Assuming that up to the 90% of the total nitrogen influent can be partitioned by the biomass resp. rejected in the UF/RO brines, this amount can then be treated in a concentrated form (> 3-4 g/L) after anaerobic digestion. The digested nitrogen can be found in organic (TKN) or inorganic (ammoniacal nitrogen) forms, of which the ammoniacal nitrogen (NH_4^+ + NH_3) usually constitutes about 80% of the TKN [237]. The ammoniacal fraction is the fraction available for final recovery by for example air stripping. Assuming an efficiency of 90% for nitrogen removal from the digestate, the final amount available for recovery is about 72% of the total nitrogen recovered after anaerobic digestion, representing 65% of the total nitrogen entering the treatment plant. If removed by air stripping, the ammoniacal nitrogen is present in the form of volatile ammonia nitrogen NH_3-N. The latter, rather than being reacted with acid solutions to produce fertilizer (such as ammonium sulphate), can instead be dissolved in a standard culture medium for microbial growth, making thus use of the dissolved reactive nitrogen as source for microbial growth. In this process chain, the reactive nitrogen would be removed from the original water matrix, recovered in a new water matrix and up-cycled directly to valuable edible microbial protein. This microbial re-assimilation of reactive nitrogen is carried out, in case of lithotrophic hydrogen-oxidizing bacteria, by using the inorganic substrates available and producible on-site. Hydrogen (electron donor), oxygen (electron acceptor) and carbon dioxide (carbon source), can be in fact found or produced directly in the used water treatment plant. In case one would use not hydrogenotrophic bacteria but normal organotrophic bacteria for the re-assimilation of the reactive nitrogen, the supply of quality electron donor

would constitute a considerable handicap which is now avoided by using on-site produced hydrogen.

6.3 The water factory driven by bio-sorptive HRAS

Biogas production from anaerobic digestion can be performed at high efficiencies when pre-concentration techniques are applied to the diluted stream. The biogas can then be upgraded to biomethane by a conventional PSA unit and fed to a fuel cell with an internal reforming unit (DFC® module). A schematic example of the water factory implementing such reforming unit is reported in Figure 6.1.

CHHP modules are implemented in full scale treatment plants, and can be therefore considered a maturing technology [152, 153]. A DFC1500™ module was described as able to convert biomethane into hydrogen, electricity and heat with an efficiency of 84% of the initial primary energy content of the fed CH_4 [153]. The specific conversion efficiencies are reported in Table 6.1. It is to note that the hydrogen production is in this case made at the expenses of the electricity produced by the module, but the residual electricity is available for on-site use. Hence, by coupling biomethane reforming and energy production with a fuel cell, hydrogen does not require any external energy supply for its production.

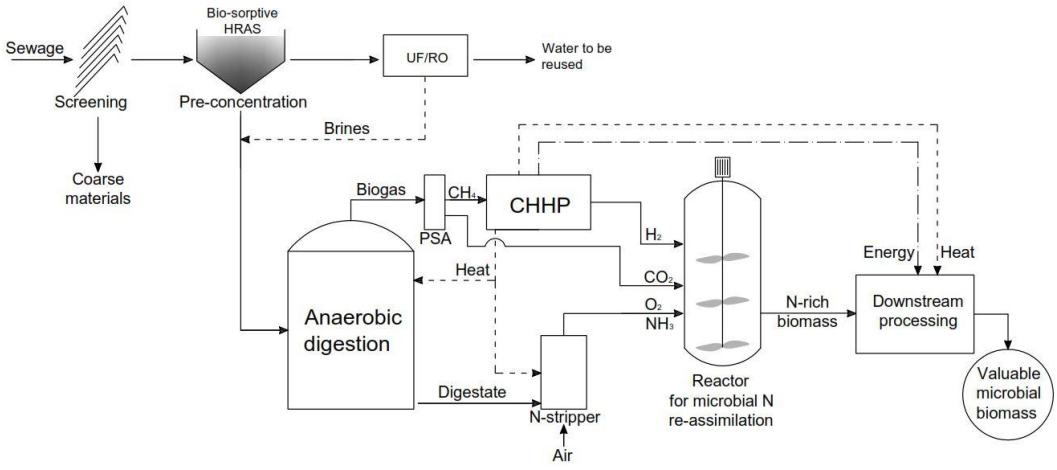

***Figure 6.1**. Scheme of the bio-sorptive HRAS based water factory upcycling reactive N to microbial biomass.*

The combination of anaerobic digestion and UF/RO brings the reactive nitrogen to levels suitable for subsequent air stripping (>3-4 g/L). The stripped nitrogen is available in the air stream in the form of ammonia, which can be supplied directly to a microbial culture reactor for nitrogen re-assimilation. Overall, anaerobic digestion coupled with

CHHP modules operating on biomethane combined with air stripping devices treating the digestate can provide all the substrates necessary for the growth of hydrogen-oxidizing bacteria: H_2, CO_2, O_2 and NH_3. Based on the amount of the available substrates calculated in Table 6.1, and on the stoichiometric requirements of hydrogen-oxidizing bacteria (4 kg H_2/kg N assimilated), the limiting stoichiometric factor for re-assimilating maximum nitrogen is hydrogen. About 11% (91 kg N) of the stripped ammonia would be re-assimilated into microbial biomass by only making use of the biogas-based hydrogen set free from the CHHP unit. In this scenario, the extra thermal energy can be used for heating the anaerobic digester as well as the ammonia stripper, providing optimal operating conditions, but can also be utilized for downstream processing (i.e. drying) of the harvested microbial biomass. Also, the produced electricity can be utilized to power both the reactor for microbial nitrogen re-assimilation and the downstream process for final biomass refining. In the broader framework of a self-sufficient water factory for on-site upcycling of recovered nitrogen, extra renewable energy for hydrogen production can be provided by wind or solar energy. Water electrolysis powered by green energy is gaining momentum e.g. in the power-to-gas concept, where electrolysis powered by solar or wind energy is used to produce hydrogen gas to be fed to the natural gas net. Relying on the efficiency of commercial modules for water electrolysis, around 48 kWh (electrolysis efficiency of 82%) are required to produce 1 kg of H_2 [238]. Therefore, according to the calculation reported above, the extra 3016 kg H_2 needed to upcycle the remaining 754 kg of recovered nitrogen could be produced on-site by installing e.g. some 6 MW wind or solar power.

***Table 6.1**. Overview of the resources recovered from the sewage by anaerobic digestion combined with CHHP.*

Influent in the treatment plant of 100 000 i.e.		Recovered after AD			Produced after CHHP		
		Product	**Amount**	**Flux**	**Product**	**Efficiency**	**Flux**
Sewage	25 000 m^3/d	Methane (after PSA)	0.14 m^3/m^3 sewage	3500 m^3/d	H_2	34.3%	365 kg/d
					Energy	41.1%	53 225 MJ/d
					Heat	8.8%	11 396 MJ/d
		CO_2 (after PSA)	30% of biogas	1500 m^3/d			
Nitrogen	1300 kg/d	NH_3 (air stripping)	65%	845 kg/d			

6.4 Conclusions and further remarks

Overall, by exploiting the intrinsic energy content of the used water, the process scheme here depicted is capable of up-cycling up to 11% of the total nitrogen recovered from the treatment plant directly to valuable microbial biomass rich in edible protein. This can be done with a rational and efficient use of the most recent techniques in used water separation (pre-concentration step), biogas utilization (CHHP) and nutrient removal/recovery (air stripping). By installing renewable energy systems such as wind energy (with very low land requirements), this extra energy can be used to produce on-site the hydrogen for up-cycling the total ammonia recovered (65% of the total nitrogen influent). Hence, anaerobic digestion can play a key role not only in energy recovery, but also in bringing nutrients and especially nitrogen recovery to a further up-cycling step.

6.5 Acknowledgments

The supervision of Prof. Willy Verstraete was essential for the development of the work reported in the present chapter.

CHAPTER

7

FROM HABER-BOSCH NITROGEN TO MICROBIAL PROTEIN: THE POTENTIAL OF INCREASING GLOBAL SUSTAINABILITY

Note:

The following chapter has been redrafted after a manuscript under preparation. The simulations with the global agricultural model MAgPIE were carried out by the Potsdam Institute for Climate Change (PIK, Berlin). The doctoral candidate, prepared the scientific description as well as the technical and economic analyses used as model input for each of the different MP pathways considered. The results of the simulations were interpreted and discussed together with the model experts, resulting in the work reported in the following chapter.

CHAPTER

7

FROM HABER-BOSCH NITROGEN TO MICROBIAL PROTEIN: THE POTENTIAL OF INCREASING GLOBAL SUSTAINABILITY

Abstract

One of the main challenges for the 21st century is to balance the increasing demand for high-quality proteins while mitigating environmental impact. In particular, cropland-based production of protein-rich animal feed for livestock rearing results in large-scale land-expansion, nitrogen pollution and greenhouse gas emissions. Here we propose and analyse the long-term potential of alternative protein supply routes based on in reactor growing of microbial protein (MP) directly from Haber-Bosch nitrogen. Our analysis revealed that by 2050, MP could replace around 13% (11–18%) of conventional feed protein demand. Of the different MP production scenarios considered, the agriculture-free and climate independent production of MP (landless MP production) appeared to be optimal since it allowed to decrease global cropland area expansion by 13% (144 Mha), global nitrogen losses by 9% (12 Mton N_r) and greenhouse gas emissions by 8% (46 Gt CO_2 equivalents). Interestingly, the technology to industrially produce microbial protein at competitive costs and thus bringing forward such major shifts is at present accessible for implementation. The boundary conditions limiting the further expansion of MP are the low cost of vegetable protein such as soybean protein and the externalization of environmental impacts of intensive conventional protein production systems. Hence, dissemination of this important potential to increase global sustainability by integrating a straightforward microbial protein production process, is urgently warranted.

7.1 Introduction

The livestock sector and the associated animal feed production through contemporary agriculture represents one of the most important contributors to global environmental pollution [239]. Two thirds of agricultural lands are used for pastures and about one third of the remaining croplands is devoted to produce protein-rich animal feed like soybeans and cereals [240]. The increasing demand for livestock products [241] leads to deforestation, global nitrogen pollution and greenhouse gas emissions. The agriculture-induced loss of reactive nitrogen (N_r) in particular has entailed a range of serious global environmental concerns including eutrophication, climate change and biodiversity loss [185, 242, 243] strongly related to the low nitrogen uptake efficiency of plant-soil systems. Indeed, about 50% of the nitrogen applied to croplands is lost through leaching, volatilisation or denitrification [244, 245]. These environmental impacts are largely driven by the production of low C/N protein-rich crops destined to feed livestock[244].

By 2050 the world's population is expected to reach approximately 9 billion people [246] a growth that combined with wealth increase will further drive the demand for animal-based proteins as part of the human diet [2]. Estimates foresee a further increase of 30 – 60% [247] and about 50% [248] in global crop production and fertilizer use, respectively. Both land expansion and intensification will have negative environmental impacts [244, 249]. Meeting the challenge of feeding the world with high quality protein, while reducing the environmental impact, requires a radical restructuring of agricultural practices. The production of protein-rich crops for the livestock industry hereby represents a key lever.

An alternative to cultivating crops to feed livestock with proteins is the production of Microbial Proteins (MP), also known as Single Cell Protein [6, 250]. MP can be produced in intensive, confined and efficient high-rate bioreactor systems using microorganisms like bacteria, yeast, fungi and microalgae [6]. Contrarily to higher plants, microbes convert reactive nitrogen into cellular proteins with an unmatched efficiency close to 100% with proteins constituting up to 70-75% of the dry biomass weight [6, 45]. In addition, protein volumetric productivity by microbes in bioreactors reaches several kg per m^3 per hour [250], which is several order of magnitude above than that can reached by higher biota. Bacteria thereby have the advantage of rapid growth on organic substrates like sugars and starch [6, 92] as well as on gaseous

substrates like methane [251] and hydrogen (with CO_2 and/or CO as carbon source) as energy and carbon source [63]. Importantly, MP is of high quality and resembles in amino acid composition that of fish meal [9].

In order enabling such a major paradigm shift where MP replaces protein rich crops in animal feed on a global scale, we considered five pathways for the production of hydrogen, methane or sugars as a substrate for MP production (Figure 7.1). Using natural gas or hydrogen generated through water electrolysis driven by renewable energy would allow for agriculture-free and virtually climate independent MP production. Alternatively, the cropland currently used for the production of low C/N protein rich crops becomes the focal point for the production of bioenergy crops, associated with much higher yields and lower fertilizer requirements. Hydrogen-based production through water electrolysis would rely on external concentrated CO_2 from industrial point sources (e.g. flue gases from power stations), which we assessed being available in sufficient quantity.

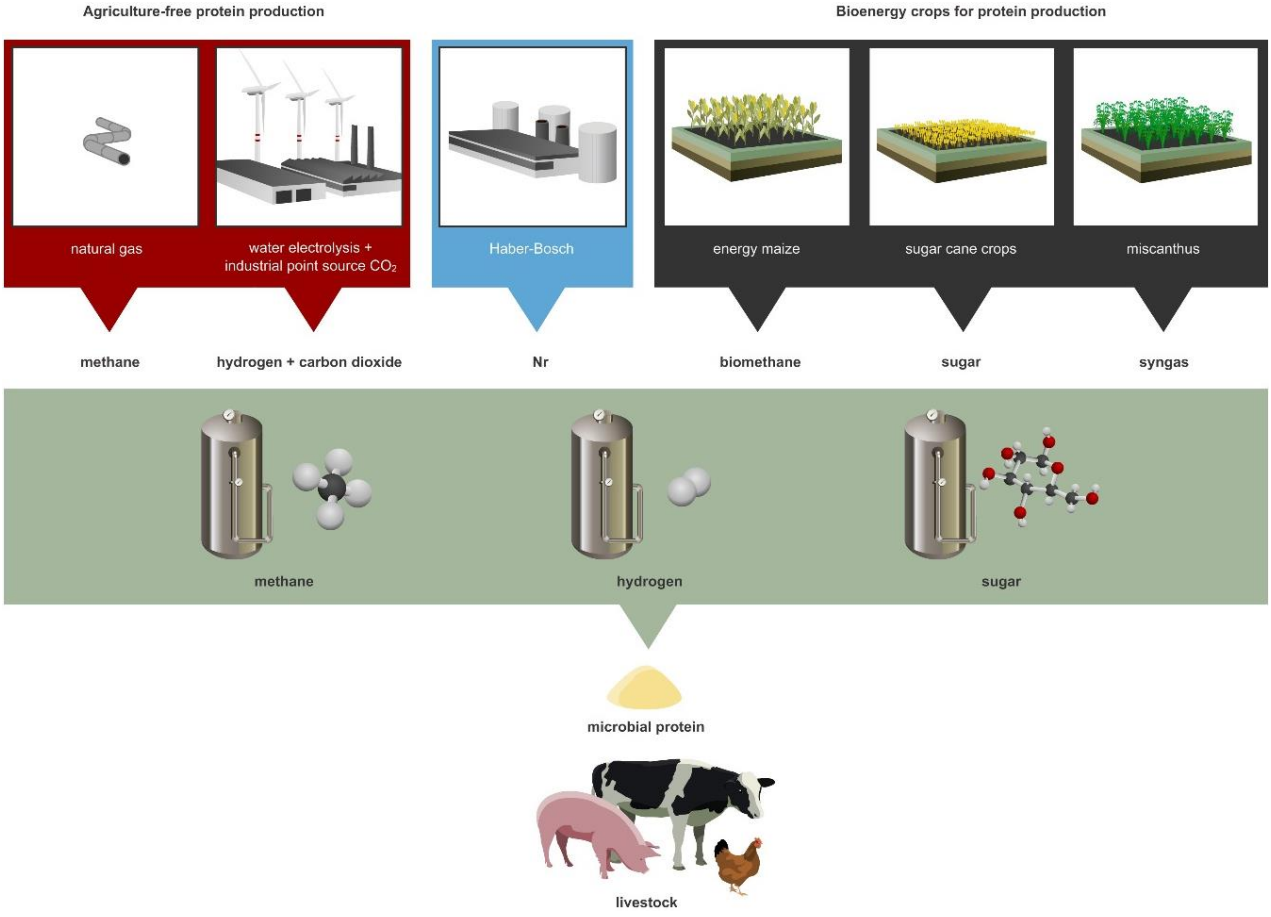

Figure 7.1. *Proposed protein supply routes for livestock production based on microbial protein (MP).*

7.2 Materials and methods

7.2.1 Technological pathways for production of Microbial Protein (MP)

In order to provide a comprehensive assessment of the impact of widespread adoption of MP as protein source in animal feed on cropland expansion, nitrogen pollution and greenhouse gas emissions, the following technological pathways for production of MP were considered:

1. Heterotrophic production of MP with raw sugar, derived from agricultural production of sugar cane, providing both the energy and carbon source required for microbial growth. From hereinafter referred to as CHMP.

2. Autotrophic production of MP using hydrogen produced by means of water electrolysis using polymer electrolyte membrane (PEM) electrolysis [252], driven by renewable energy. The carbon source originates from CO_2 point sources (e.g. flue gases from power stations). From hereinafter referred to as LLMP.

3. Autotrophic production of MP using hydrogen as energy source from syngas produced by means of biomass gasification. The syngas would also provide CO_2 used as carbon source. Agricultural production of *Miscanthus* spp. (flowering plant in the grass family Poaceae) as high C/N crop was used as source crop for gasification. From hereinafter referred to as HOMP.

4. Methylotrophic production of MP using methane oxidizing bacteria using natural gas, providing both the carbon and energy required for microbial growth. From hereinafter referred to as LLMP.

5. Methylotrophic production of MP using methane oxidizing bacteria using biogas produced by anaerobic digestion of biomass, providing both the carbon and energy required for microbial growth. Agricultural production of energy maize was used as source crop for the digestion step. From hereinafter referred to as MOMP.

7.2.2 Carbon and energy requirements of the technological pathways for MP production

7.2.2.1 Production of microbial protein using hydrogen as energy source

The section below describes the requirements in terms of renewable energy and CO_2 point sources needed to produce MP via water electrolysis using polymer electrolyte membrane (PEM) electrolysis and the amount of biomass to obtain hydrogen via biomass gasification.

Hydrogen generation by means of PEM electrolysis

In the case hydrogen is produced by means of water electrolysis, a crucial factor is the availability of technically exploitable CO_2 from (industrial) point sources (e.g. flue gases from power stations). In a recent study of the International Panel for Climate Change (IPCC), it was found that by 2050 the CO_2 capture potentials are estimated at 4.9 to 37.5 $GtCO_2$ per year (1.3 – 10 GtC) [253]. Considering an average C/N of 5 for MP production [45], the latter would be enough to produce 2.6 – 20.2 Gt of MP, while our simulations show that 419 – 552 Mton MP can be replaced in 2050 (Supplementary Materials excel spreadsheet). It is therefore paramount that there is sufficient technically exploitable CO_2 available to produce MP through PEM electrolysis.

Availability of renewable energy

Recent estimates by the International Energy Agency indicate installed capacities for wind and solar energy to increase to 2700GW and 4670GW by 2050 (Hi-Ren scenario), respectively [254, 255]. In the scenario where all MP is produced solely by means of hydrogen as energy source delivered through PEM electrolysis, the maximum amount of renewable energy needed would be 1256 –1655 GW (in order to produce 419 – 552 Mton MP). Hence, this represents 18 – 22% of the total estimated combined installed wind and solar energy in 2050, respectively.

Hydrogen generation by means of biomass gasification

We assumed an biomass-to-hydrogen yield of 0.1 kg H_2/kg biomass [256]. Note that yields during gasification found in literature are as high as 0.127 kg H_2/kg biomass [257]. A yield of 0.1 kg H_2/kg biomass corresponds to 5.5 ton of dry biomass being

necessary to produce 1 ton MP (see Table S3 in Appendix). In our simulations, we considered the use of Miscanthus as biomass substrate for the gasification process. Note that a variety of other biomass substrates are suitable for this purpose [258].

The overall biomass gasification reaction stoichiometry is as follows [259]:

$$biomass + O_2 + H_2O\ (steam) \rightarrow CH_4 + CO + CO_2 + H_2\ + H_2O + C + tar \qquad (1)$$

The formed methane will subsequently follow a steam reforming reaction:

$$H_2O + CH_4 \rightarrow CO_2 + 4H_2 \qquad (2)$$

The overall ratio of H_2:CO_2 obtained during biomass gasification is thus 2.5. With stoichiometric requirements of H_2:CO_2 for the production of MP of 5.29:1 (see Table S10 in Appendix), it is evident that there is sufficient CO_2 is available for incorporation into MP.

7.2.2.2 Production of microbial protein using methane as energy source

Natural gas

Natural gas from the grid offers a readily available energy and carbon source for MP production at virtually any place. Assuming a methane utilization efficiency of 80%, the production of 1 ton MP requires 1767 Nm^3 methane. As such, in order to produce 419 – 552 Mton MP, 768 – 975 G Nm^3 is needed. The latter corresponds to 23 – 28% of the current global natural consumption [260].

Production of high C/N crops for biogas production

We considered the agricultural production of *energy* m*aize* as substrate for the production of biogas by means of anaerobic digestion. Considering a dry matter content of 30%, we assumed a yield of 315 Nm^3 methane per ton of dry maize [261]. The latter yield translated to 5.6 ton of dry maize needed to produce 1 ton MP (see Table S4 in Appendix). Note that also other energy crops such as switch grass, clover grass, alfa alfa, sunflower and Miscanthus can be used for this purpose [262, 263].

7.2.2.3 Heterotrophic production of microbial protein using sugar cane as energy source

We considered the agricultural production of sugar cane to produce raw sugar as the required energy and carbon source to drive heterotrophic MP production. Besides raw sugar, the processing of the sugar cane yields also other by-products such as bagasse, and molasses which can either be used as livestock feed [264], or further fermented to produce MP [265, 266]. Other possibilities are offered by the anaerobic digestion or the gasification of such substrates, to produce bio-methane and syngas respectively [267, 268]. In our simulations, we have focused exclusively on the use of raw sugar as substrate for MP production, without accounting for the use of other by-products as livestock feed or for further MP production. Considering a raw sugar conversion factor of 14 ton sugarcane/ton raw sugar, and a DM content of 30% [269], the amount of sugarcane necessary to produce 1 ton MP is 4.3 ton of sugarcane (see Table S4 in Appendix).

7.2.3 Model simulations with the Model of Agricultural Production and its Impact on the Environment (MAgPIE)

The projections of the future potential environmental impacts of widespread adoption of the MP production pathways were made using the Model of Agricultural Production and its Impact on the Environment (MAgPIE). A detailed description of the MAgPIE model including its equations is available at *https://redmine.pik-potsdam.de/projects/magpie/wiki/*.

7.2.3.1 Implementing MP as secondary product within MAgPIE

In this study, the MAgPIE model was extended by a new product category, namely MP. The simulated scenarios differ in respect to three dimensions:

a) The dominant MP technology. The LLMP scenarios produce MP on the basis of hydrogen/methane and Haber-Bosch synthesized reactive nitrogen. Here, MP production requires no agricultural feedstock. The hydrogen oxidation scenario HOMP uses as reference crop the fast-growing cellulosic grass Miscanthus [270] for MP production, requiring 5.6 tons of Miscanthus per ton MP. The organic carbon oxidation pathway CHMP requires 4.4 tons of sugarcane for each ton MP. Finally, the methane

oxidation scenario MOMP requires for the production of one ton MP 5.6 tons of forage crops (see Table S3 in Appendix). These forage crops include a mixture of whole-plant silage crops like maize, alfalfa, clover, or rye grass, that are currently mainly cultivated for feed production. In comparison to maize cultivars that are grown primarily for the grain harvest, these whole plant forages resemble the high-biomass cultivars for biogas.

b) We used two different assumptions for the feed substitution potential of MP within livestock feed baskets. MP is a highly valuable feed ingredient that mainly replaces the protein part of the feed baskets, while it is a limited substitute for the feed components that provide starch or fiber for digestibility. Based on our literature survey (see Table S1 in Appendix), we defined a conservative (low) and a more ambitious (high) level of replacement for MP in feed baskets that can be attained without reducing animal productivity (see Table S1 in Appendix). The high feeding ration scenarios HF assume the upper value of the range, the low feeding ration scenarios LF the lower end of the range. It was assumed that MP primarily replaces the protein-rich oil crops and oilcakes (e.g. soy meal and cereals). If less than the potential MP replacement rate can be realized by substituting oil crops and cakes, also parts of the grain ration can be replaced. Substitution of feed was based on equal protein content. Crop residues, forage crops, pasture, molasses and other feed items were assumed to be not replaceable by MP.

c) Finally, all simulations were done within the context of three different socio-economic storylines for the general development of the agricultural sector, which were based on the Shared Socio-economic Pathways (SSPs) SSP1, SSP2, and SSP5. SSP1 describes a world with green growth, dematerialized lifestyles, global cooperation and functioning institutions. SSP5 represents a world characterized by fossil-fuel driven growth and rapid technological progress, but material-intensive lifestyles. SSP2 is the “middle-of the road scenario”, which assumes largely a continuation of current trends. Relevant in the context of this study are the different assumptions with respect to the dietary developments in SSP1, SSP2 and SSP5, with much higher consumption of animal-based products and more household waste in SSP5. Both SSP1 and SSP5 project a rapid intensification of the livestock sector. Yet, while SSP1 mainly assumes a rapid improvement in feeding efficiency in developing countries, SSP5 also assumes continuous and fast intensification in developed regions. The employment of MP is consistent with the techno-optimistic storylines.

Finally, while the nitrogen uptake efficiency in crop production is assumed to improve in all scenarios, the advances are highest in SSP1, reducing Nr losses to the environment.
In total this results in 27 scenarios (4x2x3 MP scenarios, plus 3 BASE scenarios (i.e. SSP 1-3, assuming no MP use).

7.2.3.2 Shared Socio-economic Pathways (SSPs)

The general outcomes and dynamics of the reference scenarios SSP1, SSP2 and SSP5 are broadly documented and discussed within the publications [271, 272], and the results are deposited in a public database (*https://tntcat.iiasa.ac.at/SspDb*).

7.2.3.3 Feed basket

There in not a single replacement rate for the application of MP in animal nutrition as substitute of other conventional protein sources. Depending on the MP source (yeast, bacteria, etc.) and animal species considered, the optimal values are found through feed trials focusing on animal wellbeing and productivity. Essential amino acids profile, chemical composition, effects on protein and energy metabolism are the main parameters considered when establishing the optimal replacement rate [9].
A literature survey was conducted comprising feeding trials of all major livestock categories (i.e. beef cattle, dairy cattle, pigs, broiler chickens and laying hen) to determine the amount of MP that can be used as replacement of protein-rich oil crops and oilcakes (e.g. soy meal and cereals) in the feed basket without compromising animal growth and welfare. The survey reported data on both ruminant and monogastric animals at different ages and growth stages fed with different levels of MP, as summarized in Table S1 (see Appendix). We would like to refer to the review study of Øverland *et al.* [273] in which the use of methane based MP in monogastric animals is evaluated and described in detail.
In addition to the literature survey focusing on the amount of MP in the feed basket from an animal well-being perspective, also the regional and livestock system-specific feed baskets were taken into account. As a result of the latter, it was found that the actual replacement rates of MP as substitute for protein-rich oil crops and oilcakes (e.g. soy meal and cereals) in the feed baskets, were in various cases lower than that

can be attained pure on the basis of animal well-being and reducing animal productivity (see Table S2 in Appendix).

7.2.4 Economic assessment of the technological pathways for production of Microbial Protein

7.2.4.1 General assumptions

Table S4 (see Appendix) lists the general assumptions used to determine the process requirement and assess the economic feasibility of the three MP platforms.

7.2.4.2 Energy source

Hydrogen

Table S5 (see Appendix) shows the levelled costs per kg hydrogen produced as well as the hydrogen production costs per ton MP produced. We have assumed that only 80% of the produced hydrogen is effectively used during MP production, the rest can still be converted into heat and power using a CHP unit providing the energy needed during the drying step. Yet, the latter aspect was not considered into the economic assessment, and the full cost of the drying step was taken into account (see Table S9 in Appendix).

Methane

We considered the use of natural gas under two scenarios: (i) a low cost estimate (i.e. \$0.17 / Nm^3), currently valid for the USA market, and (ii) a higher cost estimate (i.e. \$0.33 / Nm^3), currently valid for the European market. Similar to the scenario using hydrogen, we have assumed that only 80% of the produced methane is effectively used during MP production, the rest is considered to be converted into heat and power using a combined heat power (CHP) unit providing the energy needed during the drying step. Yet, the latter aspect was not considered into the economic assessment, and the full cost of the drying step was taken into account (see Table S9 in Appendix).

Raw sugar

Table S4 (see Appendix) shows price of raw sugar per ton raw sugar purchased, whereas in Table S9 (see Appendix) the costs are expressed as the cost per ton MP produced.

7.2.4.3 Other process requirements

Nitrogen and phosphate requirements

The amount of nitrogen and phosphate required and their associated operational costs are presented in Table S6 (see Appendix). The production of MP also requires the use of certain trace elements and micro-nutrients for optimal growth as well as pH regulation by means of acid and caustic dosing. We have not calculated these costs in detail as they only represent a small fraction of the overall operational expenditure and have been assumed to be part of the overhead (see Table S9 in Appendix).

Oxygen requirements

Independent of the carbon and energy source used, the reaction requires oxygen. Table S7 (see Appendix) shows the estimated operational expenditure for generation of industrial grade pure oxygen for the different pathways. Based on an oxygen utilization efficiency of 80%, it is assumed that 80% of the generated oxygen is effectively used within the MP reactors, with the other 20% being lost due to gas transfer limitations.

Pumping and mixing energy

Biological oxidation of different substrates for MP production is carried on in bioreactor equipped with mixing and pumping devices. Besides the reactor setup implemented in the fermentation step, the carbon and energy source used greatly affect the mixing energy needed, with gaseous substrate requiring stronger mixing in order to facilitate the gas-to-liquid transfer and obtain high volumetric productivities (3-4 kg MP/m^3·h). Pumping energy is instead comparable for the different substrates considered. For large scale fermentation bioreactors the mixing energy ranges between 0.2-3 kWh/m^3,

with 0.8 kWh/m^3 considered as a good average value [274]. In our assessment, we have used a rather conservative value of 1.5 kWh/m^3, taking into account the energy demand of both mixing and pumping devices, independent of the technological pathway considered.

Dewatering, drying and sterilization of MP

In order to guarantee a high-quality, sterile and dry product, the MP produced in the fermentation reactor, is subjected to a heat-treatment to increase the protein accessibility (by cell lysis), reduce the nucleic acid content and obtain a dry sterilized product [275]. Prior to heat-treatment, the MP is dewatered to a dry solids content of around 25% by means of a centrifuging step, allowing to reduce the energy requirements of the heat-treatment step. For the drying step, a spray drying unit with an integrated fluidised bed is proposed. The use of spray drying with an integrated fluidised bed is common practice in many drying processes in the food processing industry [276]. The operational costs of drying and sterilization are mainly related to the energy consumption of the process (see Table S8 in Appendix).

7.3 Results and discussion

In order to assess the environmental impact of the implementation of these MP production pathways on cropland expansion, nitrogen pollution and greenhouse gas emissions, we conducted comprehensive model simulations until the year 2050 using the Model of Agricultural Production and its Impact on the Environment (MAgPIE) [244, 249]. To enable a comprehensive assessment we considered three Shared Socioeconomic Pathways (SSPs) [277] (i.e. SSP1 (“Sustainability”), SSP2 (“middle-of-the-road”) and SSP5 ("Fossil Fuel Development")) and three MP feed replacements rates.

We conducted a literature survey to determine the amount of MP that can be used as protein source without negative consequences on animal productivity and wellbeing (see Table S1 in Appendix). We considered MP only to replace currently used protein concentrates in animal feed baskets (e.g. soy bean meal, oilcakes, oil crops and pulses). Our simulations indicated that globally up to 234 (179 – 307) Mton of MP could substitute concentrated protein feeds in the livestock sector, only comprising 2% (2–

4%) of the total dry matter feed demand in 2050, equivalent to 13 (11–18%) of feed protein because of the high protein-content of MP.

These marginal changes to the livestock feed baskets (see Table S2 in Appendix) however would have in most scenarios a substantial global environmental impact. For SSP2 (“middle-of-the-road”) with medium protein replacement rates, the agriculture-free and climate independent production of MP would result in the largest decrease in global cropland expansion a 144 Mha (13%). This scenario would also lower the global nitrogen losses from croplands by 12 Mton N_r (9%) and 46 Gt CO_2 equivalents (8%) due to the decrease in emissions deriving from cumulated land-use and land-use change (LULUC) in the period between 2005 and 2050. However, this production route would require 413 billion Nm^3 of natural gas ~12% of the current global natural gas consumption), thereby reducing its viability as a long-term sustainable solution. In case hydrogen is generated through water electrolysis it would require 702 GW of electricity, which equals to ~10% of the estimated combined installed solar and wind energy by the year 2050 (Hi-Ren scenario)[278].

Natural gas or electricity needs are avoided when bioenergy crops are used as energy and carbon source via syngas, sugar or biogas (see Figure 7.1). To provide the same amount of MP, this would require 1287 Mt of Miscanthus, 1006 Mt of sugarcane or 1310 Mt of bioenergy forage crops. The consequences for land-expansion, nitrogen pollution and GHGs would be still substantial, yet lower than for agriculture-free production (see Figure 7.2).

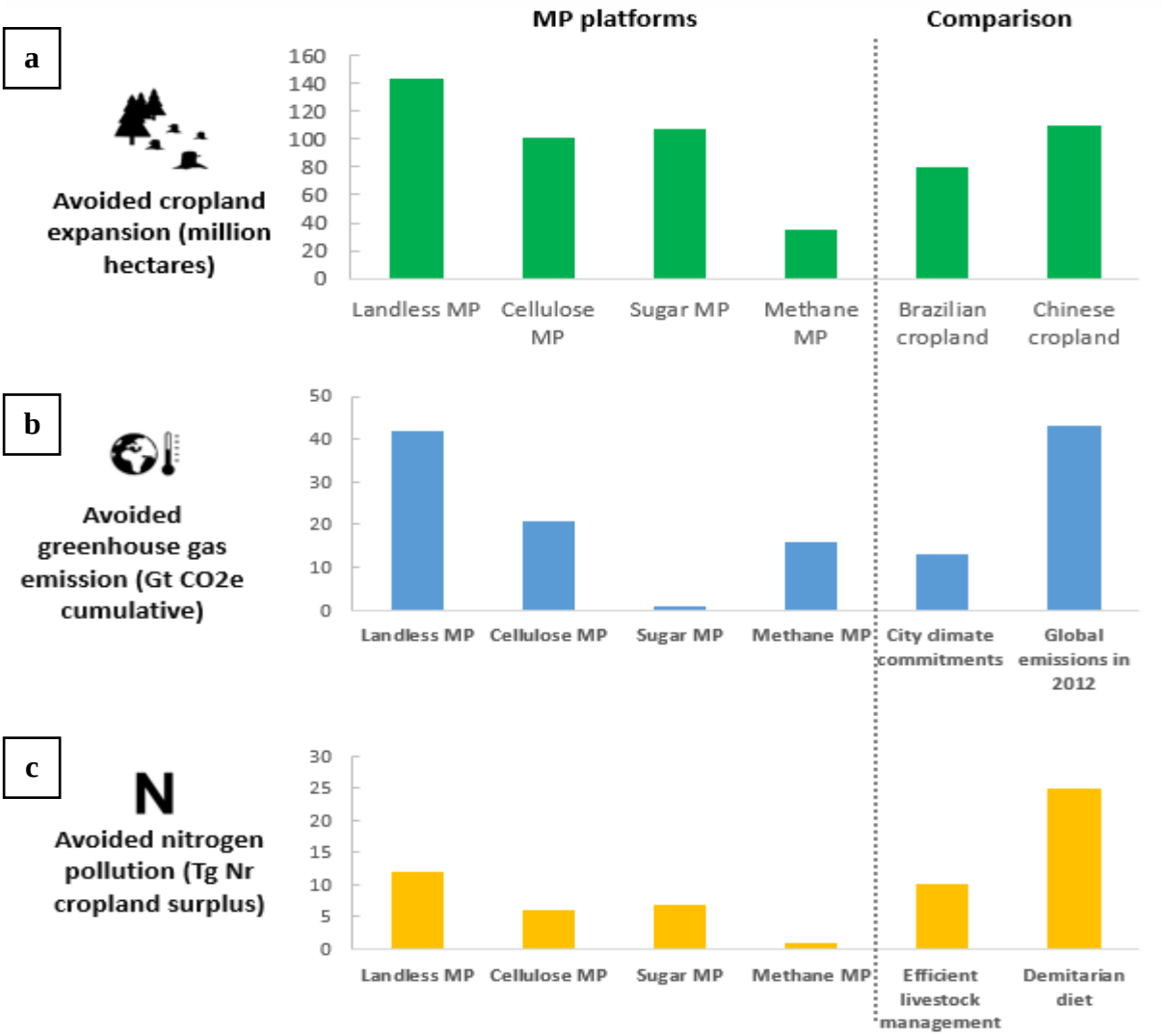

Figure 7.2. *Feeding microbial protein (MP) to animals can substantially decrease global cropland expansion, greenhouse gas emissions and nitrogen pollution. The impact of microbial protein on global (a) cropland expansion until 2050, (b) cumulative greenhouse gas emissions until 2050 and (c) nitrogen pollution in 2050 relative to the baseline scenario without MP. For comparison, we use land-areas* [279]*; carbon budgets of city climate commitments for 2050 (involving 228 cities and 436 million people) and global emission in 2012; nitrogen mitigation potentials of improved livestock management and demitarian diet (i.e. a balanced diet with reduced meat consumption)* [258]*.*

The replacement of feed by MP, as well as the substrate cultivation for MP production would restructure the agricultural supply chain and the nitrogen flows considerably. The implementation of the sugar-to-protein pathway, for example, would cause the Haber-Bosch nitrogen fertilization to drop by 14 Mton Nr (-10%). The quantity of harvested nitrogen from crops and crop residues would then drop by 25 Mton N_r (-16%) and 4 Mton N_r (-4%), respectively. In addition, by replacing large amounts of

soybean by MP, the global biological fixation would also substantially decrease by 18 Mton (-22%).

In addition to benefits reported here, the MP approach is also expected to diminish the use of phosphorus (another essential nutrient for crops used in large amounts) [280], fresh water for irrigation [281] and the use of pesticides [282]. Furthermore, by-products from sugarcane processing such as bagasse and molasses can be beneficially used as livestock feed [264] or as substrate for fermentation and production of syngas [266, 268].

Replacing agricultural-based animal feed with MP is not hypothetical. MP is an officially recognized and approved commercial feed ingredient for all livestock [283]. In fact, MP production from methane was already achieved at industrial scale in the 70's [8]. Comparatively low market prices of conventional agriculture-based animal feed, the relatively under-developed fermentation technology and limited focus on resource efficiency limited its success. The recent progress in the domains of in industrial biotechnology, microbial engineering, and process and reactor technology has reduced the cost of MP production with methane based MP production already commercially available [284]. Our assessment shows the economic potential of other MP production routes (see Table S8 in Appendix). Hydrogen produced by means of water electrolysis could be a promising technology when using off-peak energy that may become available under a higher penetration of electricity grids by renewable energy (see Table S8 in Appendix).

Very important, in recent years the large negative economic, environmental and social externalities of agricultural practices on e.g. climate change, human health and ecosystem functioning have become evident and have been estimated as high as 0.3 – 3.0% of the global gross domestic product (~$225 – $2250 billion dollar) annually for reactive nitrogen losses alone (see Table S2 in Appendix). Until now, these negative externalities have not been sufficiently priced in markets. Their internalization would constitute a powerful driver for the economic potential of MP in terms of the overall costs for the society at large.

7.4 Conclusions

Overall, our results show that production of MP can alleviate a set of critical limitations in the agricultural food supply chain by decoupling livestock production from land-based production of protein-rich animal feed. Substantial global environmental benefits can already be achieved with only minor changes to animal diets. Clearly, the widespread adoption of MP as a "stand-alone" solution will not be sufficient. It will also require improvements in current livestock and manure management, fertilization practices and in particular changes in human dietary patterns. Furthermore, the development of technologies that can effectively recover used reactive nitrogen [13] as well as the reuse of organic wastes such as agricultural by-products as carbon and energy source [285] are essential to further reduce the environmental impact. Ultimately, all the above are needed to ensure feeding future generations with high-quality proteins in a sustainable way. Achieving major environment benefits by a simple and marginal change in animal diets is a good start.

7.5 Acknowledgements

We acknowledge Dr. Benjamin Bodirsky, Prof. Alexander Popp, Dr. Florian Humpenöder and Dr. Isabelle Weindl for working on the model simulations and its interpretation as well as for contributing meaningfully to the manuscript reported in this chapter. Dr. Ilje Pikaar is also acknowledged for his work on the economic calculations and the essential collaboration during the writing of the manuscript here presented.

Appendix

Table S1.

Feed substitution potential of MP within livestock feed baskets.

Type of livestock	MP replacement in feed baskets	refs	Remarks
Pigs	21% (dry matter basis)	[279]	In this study, feeding trials using 24 pigs (with a mean body weight 44 kg) were used to determine the digestibility of energy, nitrogen and amino acids. MP was added to the basal diet (21% dry matter basis) as such that the overall crude protein content of the feed basket remained constant at 18±0.5% crude protein on a DM basis. The inclusion of MP was compared with the addition of fish meal added to the basal diet at 24% (dry matter basis). Pigs fed with MP were found to have higher net protein utilization rates and apparent and true digestibility of amino acids than the pigs fed with fish meal.
	10% – 20% (dry matter basis)	[280]	In this study, the impact of 10% and 20% inclusion of MP in diets offered to 27 piglets from 21 – 42 days of age and compared with diets with fish meal. The diets were characterized by a slight increase in overall crude protein content of the feed baskets from 21±1% to 27±1% crude protein content on a dry matter basis when increasing the MP replacement share from 10 to 20%, respectively. No difference in growth rate was observed with the piglets converting equally efficient as piglets offered diets containing fish meal.
	5%, 10.5%, 16% (dry matter basis)	[281]	In this study, soy bean meal was replaced in basal diet (which comprised of soy, wheat and barley) by MP (methane based production of bacterial protein) at three replacement rates (i.e. 5%, 10.5% and 16% of DM basis) as such that the overall crude protein content of the feed basket remained constant at 22±0.5% crude protein (DM basis). No increase in plasma concentrations was observed, indication the absence of any uricogenic effect.
	4%, 8%, 12% (dry matter basis)	[282]	In this study, the effects of replacing fish meal, soy bean meal, and meat and bone meal in conventional diets with MP (methane based production of bacterial protein) at a replacement rate of 4%, 8% and 12% on the growth of weanling pigs examined. Similar growth performance to that obtained with a conventional diet was observed. Note that that the overall crude protein content of the feed basket within the different feed

			baskets remained constant at 23±0.5% crude protein on a DM basis.
	5%, 10%, 15% (dry matter basis)	[290]	Feeding trials were conducted to investigate the effects of increasing the dietary content of MP (methane based production of bacterial protein) on the protein and energy metabolism of pigs from weaning to a weight of 80 kg. Soya-bean meal was replaced with MP at a replacement rate ranging from 0 – 15% (DM basis) as such that the overall crude protein content of the feed basket remained constant at 21.8±0.7% crude protein content on a DM basis. Both the overall protein and energy metabolism in growing pigs were not affected in all MP replacement rates used.
	17%, 20%, 35%, 40%, 52%, 60% (digestible N basis)	[291]	In this study, feeding trials were conducted in which experimental diets contained increasing levels of MP (methane based production of bacterial protein) as replacement of soybean meal as such that the overall crude protein content of the feed basket remained constant at 38.3±0.5% crude protein content on a DM basis. It was found that MP was a suitable protein source without any relevant difference compared to the soybean meal diet.
	6%, 12%, 15% (dry matter basis)	[292]	In this study, it was found that replacement of soy beans by MP at replacement rates up to 12% (dry weight basis) in diets for pigs from 26 kg live weight until slaughter had no adverse effect on their overall growth performance as well as carcass lean or fat content. However, MP levels of up to 15% reduced growth rates during the piglet period and increased carcass fat content. The latter was found to be caused by marginal dietary lysine levels. Overall, it was concluded that the addition of MP achieved a dose dependent improvement in the utilization of total amino acids and lysine and the quality of back fat determined as fat firmness and fat color. Note that that the overall crude protein content of the feed basket within the different feed baskets remained constant at 20±0.7% crude protein on a DM basis.
	5%, 10%, 15% (dry matter basis)	[293]	In this study, MP replacement rates and dietary compositions were used according to (*57, 59*). It was found that the inclusion up to 15% MP on a dry matter basis did not affect the metabolic function, as reflected in the measured blood parameters.
	5%, 10%, 15% (dry matter basis)	[294]	In this study, feeding trials were conducted in which experimental diets contained increasing levels of MP (methane based production of bacterial protein) as replacement of soybean meal. The inclusion of MP was balanced by a reduction in amount of soybean in the diets in order to maintain constant crude protein

			levels within the different diets. Pigs fed diets containing MP had reduced thiobarbituric acid reactive substances (TBARS) value in backfat and muscle, reduced intensity of odor and rancid odor and taste in pork after short-time storage, and reduced off-odor and off-taste after intermediate-time storage. To conclude, adding MP to diets for pigs changed the fatty acid profile, improved the oxidative stability, and sensory quality of pork.
	23% (dry matter basis)	[295]	Feeding trials were conducted to determine the digestibility of MP grown on methane. MP was added at a replacement rate of 23% to the basal diet. Total tract apparent digestibility and the ileal nitrogen digestibility were found to be 85.4% and 78.1%, respectively.
In the MAgPIE model simulations, we used 8 – 15% of MP replacements shares to the feed baskets for pigs as default feeding (DF) and ambitious feeding (AF) ratio, respectively. Replacement shares are indicated on dry matter basis of the total feed basket.			
Beef and dairy cattle	0, 10, 20% (dry matter basis)	[296]	During feeding trials over a period of 32 weeks it was found that replacing groundnut oil meal with up to 20% MP as protein source in the feed baskets had no detrimental effects on performance in calves.
	0%, 5%, 7.5% (dry matter basis)	[297]	Feeding trials using veal calves were conducted using MP at replacement rates of 2.5, 5 and 7.5% as a replacement for milk protein as such that the overall crude protein content of the feed basket remained constant at 25.5±0.7% crude protein content on a DM basis. Similar growth rates during the course of the fattening period (i.e. increase in weight of the veal calves from 60 – 150 kg) at all MP replacement shares.
	0, 5, 10, 15% (dry matter basis)	[298]	Feeding trials were carried out to determine the impact of yeast, grown on methanol, as a protein source using 4 – 5 months old fattening lambs. Similar performance was observed amongst the range of yeast inclusion tested.
	0%, 10.2% and 22.9% (dry matter basis)	[299]	In this study, the impact of inclusion of yeast derived MP from sugar cane, as a replacement of soy bean, on dry matter intake and digestibility, milk production and quality was examined in dairy goats. During the 90 day feeding trial, it was found that MP can be used as a protein supplement without any differences in milk production and quality observed. The different diets were characterized by a constant crude protein content of 23.8±0.9% on a dry matter basis.

	Not reported	[300]	The replacement of groundnut-cake protein with MP significantly (P<0.05) increased the milk yield in lactating dairy goats without observing any changes in the milk composition.
	8% (dry matter basis)	[295]	In 1995 the European Union approved the use of bacterial protein grown on natural gas as protein source in veal calves up to a maximum replacement share of 8% (Council Directive No. 82/471/EEC).
In the MAgPIE model simulations, we used 8 – 15% of MP replacements shares to the feed baskets for beef cattle and dairy cattle as default feeding (DF) and ambitious feeding (AF) ratio, respectively. Replacement shares are indicated on dry matter basis of the total feed basket.			
Broiler chicken and laying hens	2%, 4%, 6%, 8%, 10% (dry matter basis)	[301]	Overall, substitution of soybean meal protein with increasing levels of MP sensibly lowered feed-to-gain ratio during the last part of the feeding period. Sensory analysis of thigh meat after 2 month of frozen storage showed that meat from 35-d-old chickens fed with inclusion of 6% and 10% MP in their diets had less odour intensity and less rancid flavour than meat from the control group. Other sensory attributes were not affected by treatment. Note that that the overall crude protein content of the different diets remained constant at 22.7±0.4% crude protein on a DM basis.
	6% (dry matter basis)	[302]	It was concluded that 6% of MP (methane based production of bacterial protein) can replace soybean meal in diets for broiler chickens without impairing growth performance. The overall crude protein content of the different diets remained constant at 26.3±1% crude protein on a DM basis.
	2%, 4%, 6% (dry matter basis)	[303]	The effects of replacing soybean meal or fish meal with 2, 4 or 6% MP on growth performance, digestibility of amino acids and sensory quality of meat, were examined using 630 broiler chickens. It was concluded that MP can replace soybean meal or fish meal protein in broiler chicken diets within the inclusion rates tested (i.e. up to 6%). Diets were characterized by a constant crude protein content of 23±1% on a dry matter basis.
	9.6%, 10%, 19.2%, 20%, 29% (dry matter basis)	[304]	It was found that the inclusion of 10% – 20% of MP as a replacement of soybean meal to 5 day-old male broiler chicks reduced growth rate and efficiency of food conversion over a 14 days period. In the same study, it was found that MP fed to chicks (with an age of 21 days) at a rate of 9.6% marginally improved growth rates, efficiencies of food utilization and nitrogen retention. Further increasing the MP inclusion rate up to 19.2 and 29% caused adverse effects. The different diets tested were characterized

			by constant crude protein contents on a dry matter basis.
	2%, 4%, 6% (dry matter basis)	[305]	In this study, the effect of the inclusion of MP as a replacement of fishmeal to basal diet with increasing concentrations (0%, 4% and 6 %) on the energy metabolism and carcass composition was investigated. It was concluded that the overall protein and energy metabolism as well as carcass composition were not influenced by a dietary content of up to 6% MP. The latter corresponds to 20% of dietary nitrogen. The different diets tested were characterized by a constant crude protein content of 24.4±1% on a dry matter basis.
	4%, 8%, 12% (dry matter basis)	[306]	In this study, the effects of the inclusion of MP (methane based production of bacterial protein) as a replacement of soybean meal to basal diet with increasing concentrations (4%, 8% and 12%) on growth performance and carcass quality in broiler chickens were examined. reduced feed intake and improved gain, but did not affect weight gain compared to the soybean meal based control diet. The different diets were characterized by a constant crude protein content of 25.5±0.5% on a dry matter basis.
	4%, 8%, 12% (dry matter basis)	[307]	In this study, the effects of the inclusion of MP to basal diet with increasing concentrations (4%, 8% and 12%) on fatty acid composition, the profile of volatiles by dynamic headspace gas chromatography-mass spectrometry, and sensory quality of frozen-stored broiler chicken thigh meat was examined. In the basal diet, containing e.g. wheat and maize, soy bean meal was used as protein source. Replacing soy bean by MP resulted in a reduction in lipid oxidation products in frozen-stored meat. The latter is important, as it is associated with quality deterioration and reduced consumer acceptance. The different diets tested were characterized by a constant crude protein content of 26.9±0.8% (DM basis).
	8% (dry matter basis)	[308]	In this study, the effects of bacterial protein meal at an 8% replacement rate (as a replacement of soybean meal) to broiler chickens (1–35 days of age) on the fatty acid composition, lipid oxidation and sensory quality on frozen thigh meat stored frozen for 6 month was examined. It was found that the inclusion of MP did not affect the sensory quality parameters, but had a positive effect in terms of reduced volatiles in frozen-stored broiler meat. The latter was hypothesized to be related to antioxidant properties of the bacterial autolysate. The different diets tested were

			characterized by a constant crude protein content of 26.5±0.85% (DM basis).
In the MAgPIE model simulations, we used 6 – 12% of MP replacements shares to the feed baskets for broiler chicken and laying hen as default feeding (DF) and ambitious feeding (AF) ratio, respectively. Replacement shares are indicated on dry matter basis of the total feed basket.			

Table S2.

Environmental costs of reactive nitrogen losses to the biosphere.

Region	Annual costs	Costs (per kg N)	ref	Remarks
China	US$9.5−31 billion	$2.1-3.8 kg NO_x-N $0.4-3.3 kg NH_3-N $0.3 kg N_2O-N	[309]	This study estimates that agriculture accounted for 95% of the NH_3 and 51% of the N_2O in china in the year 2008. In the same study it was also estimated that the total atmospheric emissions of reactive nitrogen causing related health damage ranged US$19−62 billion per year. Of this number, agricultural induced emissions accounted for more than 50% of the costs.
EU	€35−230 billion	€10-30kg NO_x-N €2-20kg NH_3-N €5-15kg N_2O-N	[310]	This study revealed that the costs of agricultural induced reactive nitrogen losses exceed the economic benefit due to increase primary crop production by a factor 4. Overall, the annual costs associated with agricultural reactive nitrogen losses was estimated to range between €35−230 billion per year.
USA	US$81–$441 billion	No information given on costs per kg N	[311]	This study is the first assessing the cost associated with reactive nitrogen losses to the biosphere from human activities in the United States. The study revealed that the total potential environmental and health economic impact of reactive nitrogen losses from anthropogenic nitrogen summed up to an average of US$210 ($81–$441) billion per year in the beginning of the 21th century. Of this, ~75% of the estimated costs were associated with agricultural induces losses.
World	US$200-2000 billion	No information given on costs per kg N	[312]	In this report, conducted by the European Nitrogen Assessment, a costing procedure based on the European situation was implemented aiming at calculating the global cost of nitrogen pollution. Taking into account that the global costs would be approximately a factor threefold of the European situation, resulting in an overall estimated costs associated with reactive nitrogen losses ranging between 200 to 2000 billion US dollars annually.

USA		\$900 (\$100 – \$59,400) ton NH_3 250 (\$20-\$1780) ton NO_x	[313]	In this study, the Air Pollution Emission Experiments and Policy (APEEP) model (an integrated assessment model) was used to determine the economic impact of air pollution by means of air quality modeling, exposure, dose-response and valuation for a large range of point sources, based on data of more than 10,000 sources measured by the US EPA.
USA		\$3.03 (\$1.25 – \$4.80) kg NH_3 14.6 (\$2.0-\$27.27) kg NO_x	[314]	This study aimed to determine the environmental and health externalities associated with the production of different agricultural crops such as corn and switch grass for the production of ethanol. While the purposed and crops used are different, the externalities are directly assessed based on the emissions of NH_3 and NO_x.
USA and EU		€3.1 – €30 kg NH_3-N (to air) €13 – €43 kg NO_x-N (to air) €5 – €54 kg Nr (to water) €2-18kg N_2O-N (to air)	[315]	In this study, the findings of several previous studies (*84-86*) on the externalities of reactive nitrogen emissions in terms of health, ecosystems/coastal systems, crop decline and climate change were summarized.

Table S3.

Overview of the technological pathways for production of Microbial Protein

MP Substrate		**ton substrate / ton MP-N**	**ton substrate / ton MP-bulk (@ 70% protein)**	**Microbial route**
High C/N crops	Maize (dry weight)	49.6	5.6	Methane oxidation
	Miscanthus (dry weight)	50.2	5.5	Hydrogen oxidation
	Sugar cane (dry weight)	34.9	4.3	Organic carbon oxidation
Natural gas	CH_4 gas	9.2	1.01	Methane oxidation
Hydrogen gas	H_2 gas	4.0	0.46	Hydrogen oxidation

Table S4.

General assumptions for the economic assessment of the MP production routes

Parameter	Value	unit	Refs.	Remarks
Electricity price	0.10	$/kWh	[316]	Price may fluctuate between regions, and may be as low as $0.05/kWh. Note that levelized costs for wind energy are already as low as $0.06/kWh.
Natural gas price	0.17-0.33	$/Nm^3	[317]	The prices used are equal to 5 and 10 $/mmbtu, respectively. 10 $/mmbtu is the current and predicted future price in Europe, whereas the current price and forecasted price in the US are lower than 5 $/mmbtu.
Sugar price	250	$/ton	[318]	2005 prices. The same report, prepared by the US department of Agriculture, it is forecasted that raw sugar price will increase to around $440 by 2025.
Soy price	450-600	$/ton	[82]	Note that soy has a protein content of around 32-40%, compared to 70-75% for MP.
Fishmeal price	1750	$/ton	[319]	The price of fish meal has been steadily rising since 2000 and is expected to double by 2030.
Market value MP	1750	$/ton		The market value of MP is estimated to be the same as fish meal as it has a similar protein and amino acid composition. It even has slightly higher protein content than fishmeal (i.e. 65% for fish meal versus 70-75% for MP)

Table S5.

Levelled costs for hydrogen production (including both CAPEX and OPEX and subsequent impact on OPEX for MP production.

Hydrogen production route	Costs ($/kg H_2)	Costs ($/ton MP)[d)]	Costs ($/ton MP-protein)[d,e)]	Remarks
biomass gasification	$1.61	902	$1288	The cost for biomass gasification are based on study of the US department of Energy [256]. The same study reports on future prediction of a further reduction in costs to $1.47/kg H_2 using the H2A production model [320]. This model includes capital, operating, maintenance, feedstock, utility, transport and replacement costs.
water electrolysis (current practice)	$5.00	2800	$4000[c)]	The costs for hydrogen production by means of PEM electrolysis are based on an electricity price of $0.05/kWh [321].
water electrolysis (future predictions)	$3.00	1680	2100	Future predicted levelled costs for hydrogen production using PEM electrolysis are predicted at $3/kg H_2 produced [321]. Note that this also includes compression, storage and dispensing, which are not needed for the production of MP.
water electrolysis (off-peak electricity)	$0.70	392	$560 [c)]	The predicted future levelled cost are $3/kg H_2 produced [321]. Considering that an electricity price of $0.05/kWh is used, the latter equals to electricity costs of $2.3/kg. As such, we estimated the levelled costs at $0.7/kg H_2. Note that this also includes compression, storage and dispensing.

a) Costs are excluding delivery, compression, storage, and dispensing costs. Important to note is that in the context of MP production, compression, storage and dispensing are not required.

b) The calculated costs per ton MP are based on stoichiometric requirements of 4 kg H_2/kg N and conversion of N to protein of 6.25 [322].

c) Additional economic benefits may derive from using CO_2 from industrial point source (e.g. reduction in carbon tax).

d) We assumed hydrogen uptake efficiencies during the production of MP of 80%.

e) The final cost is here expressed in terms of cost per ton of protein (70% protein content of the final MP product).

Table S6.

Nitrogen and phosphorus requirements and for MP production and associated operational costs.

Parameter	value	unit	Remarks
NH_3-N required	0.112	ton NH_3-N/ton MP	Based on a conversion of nitrogen-N to protein of 6.25 [322, 323], at an average protein content of MP of 70% on a dry weight basis.
Price nitrogen	800	$/ton N	Food-grade ammonia nitrogen is used.
Costs NH_3-N per ton MP produced	89.6	$/ton MP	
Phosphate required	32	kg PO_4 /ton MP	
Price phosphoric acid	1000	$/ton	Food grade phosphoric acid used.
Costs phosphate per ton MP produced	32	$/ton MP	

Table S7.

Oxygen requirements and OPEX of generation of industrial grade oxygen.

Parameter	Value	Unit	Remarks
Oxygen requirements hydrogen driven MP production (@70% protein)	2.05	kg O_2/kg MP	Based on equation 1, Table S10.
Oxygen requirements methane driven MP production (@70% protein)	2.50	kg O_2/kg MP	Based on equation 2, Table S10.
Oxygen requirements heterotrophic MP production (@70% protein)	0.85	kg O_2/kg MP	Based on equation 3, Table S10.
Industrial grade oxygen generation	0.05 (0.04-0.06)	\$/m^3 O_2	Based on [324] and an electricity price of \$0.10/kWh
Industrial grade oxygen generation	35 (28-42)	\$/ton O_2	
% percentage effectively used	80%	%	
Cost O_2 hydrogen based MP production	90	\$/ton MP	
Cost O_2 methane based MP production	109	\$/ton MP	
Cost O_2 heterotrophic based MP production	37	\$/ton MP	

Table S8.

Energy requirements dewatering, sterilization and drying.

Parameter	Value	Unit
Energy requirement centrifuge	700	kWh/ton MP
Electricity price	0.10	$/kWh
Dry solids content MP after centrifuge	25%	wt%
Energy costs per ton MP dried	70	$/ton MP
Energy requirements spray-drying [a)]	3500 [286]	MJ/ton H_2O
Energy content natural gas	38.7	MJ/m^3
Natural gas required	90	m^3/ton H_2O
Price natural gas	0.33 (0.17-0.33) [317]	$/$m^3$
Dry solids content final product	100%	wt%
Energy requirements spray drying per ton MP dried	8167	MJ/ton MP
	211	m^3 CH_4 / ton MP
Energy costs per ton MP dried	$90	$/ton MP

a) Spray drying integrated with a fluidized bed was considered as reference method for the drying step. The latter is a commonly applied method for drying in the food industry [286].

Table S9.

Summary of operational expenditure for the various MP production routes.

MP platform	Substrate	Substrate[a]	O_2	CO_2	Mixing - pumping	Dewatering	Drying / sterilization	Nitrogen and Phosphate	Overhead (100% of CAPEX annuity)	$OPEX_{total}$ (70% protein) [$/ton MP]	$OPEX_{total}$ (100% protein) [$/ton MP]
High C/N crops	Methane (after energy maize anaerobic digestion)	$831	$109	-	$38	$70	$90	$149	$270	**$1.557**	**$2.224**
	Hydrogen (cellulose gasification (current price scenario))	$886	$90		$38	$70	$90	$149	$270	**$1.592**	**$2.274**
	Hydrogen (biomass gasification (target price scenario))	$666	$90		$38	$70	$90	$149	$270	**$1.372**	**$1.960**
	Sugar cane (dry weight)	$504	$37	-	$38	$70	$90	$149	$270	**$1.158**	**$1.655**
Natural gas	CH_4 gas (low price scenario)	$300	$109	-	$38	$70	$90	$149	$270	**$1.027**	**$1.466**
	CH_4 gas (high price scenario)	$583	$109	-	$38	$70	$90	$149	$270	**$1.309**	**$1.870**
hydrogen	H_2 gas (water electrolysis - standard price)	$1650	$90	$117	$38	$70	$90	$149	$270	**$2.590**	**$3.700**
	H_2 gas (water electrolysis - off peak energy)	$385	$90	$117	$38	$70	$90	$149	$270	**$1.325**	**$1.893**

a) Note that we have assumed a utilization efficiency of hydrogen and methane of 80%, which is included in the prices listed here.

Table S10.

Stoichiometry of MP production normalized per mole of biomass (anabolic products) using the different pathways.

Microbial route	Reaction stoichiometric	Anabolic products (mol)	Catabolic products (mol)	References
Hydrogen oxidation	$21.36H_2 + 6.21O_2 + 4.09CO_2 + 0.76NH_3$	$C_{4.09}H_{7.13}O_{1.89}N_{0.76}$	$18.70\ H_2O$	[63]
Methane oxidation	$7.59\ O_2\ + 6.13\ CH_4\ + 0.76\ NH_3$	$C_{4.09}H_{7.13}O_{1.89}N_{0.76}$ [a]	$2.69\ g\ CO_2$	[251]
Organic carbon oxidation	$0.67\ C_{12}H_{22}O_{11} + 3.00\ O_2 + 1.00\ NH_3$	$C_5H_7O_2N$	$3.00\ CO_2 + 5.33\ H_2O$	[326]

[a] The same biomass composition reported for autotrophic hydrogen MP production is assumed; an equivalent amount of nitrogen was included in the stoichiometry for biomass formation from methane.

CHAPTER 8

GENERAL DISCUSSION AND PERSPECTIVES

CHAPTER 8

GENERAL DISCUSSION AND PERSPECTIVES

8.1 Microbial biotech

8.1.1 Research outcomes

Currently, microbial fermentation technologies are mainly based on strict axenic conditions, requiring energy intensive sterility measures, often resulting in the discontinuation of the process for handling procedures. Innovative processes able to guarantee a stable and constant composition of the biological system without the need to constantly sterilize process equipment can result in a consistent decrease of operating costs in microbial biotechnology.

The approach adopted in the present thesis demonstrates the possibility of enriching a generic microbial culture with very effective actuators (HOB) constituting the core of a microbiome, coexisting with satellite species, carrying out secondary functions. More specifically, after an initial enrichment, the modification of process conditions towards low hydraulic and solid retention time under continuous reactor conditions, allowed selecting a highly competitive microbial culture, performing stably along several months of experimentation. Such conditions are indeed common in fermentation technology, yet in the present work no strict sterile conditions were adopted.

The resilience of the microbial consortium should be tested against the invasion of specific pathogenic bacteria or predatory microorganisms, which could undermine the stability of the biological system. Yet, if compared to current fermentation processes, the fact that the culture was stable for months of operations represents already a remarkable achievement.

The highly enriched microbiome was indeed characterized by the constant dominance of the *Sulfuricurvum* genus at relative abundances above 95%. Such genus, known to

oxidize hydrogen under microaerophilic conditions, was yet never studied and characterized under fully aerobic high rate fermentation processes. The remarkable similarity of kinetic parameters such as maximum biomass yield and growth rate to other known pure cultures of HOB allows considering also the *Sulfuricurvum* genus amongst the best performing HOB. The coexistence with small percentages of other heterotrophic bacteria leads to a straightforward comparison with the methane based platform, where a bath of *Methylococcus capsulatus* is characterized by the constant presence of 2-3% of other heterotrophic bacteria. In the latter case though, such stable microbial configuration was obtained by means of an unwanted external invasion, eventually providing beneficial effects to the overall bioprocess performances. In the work here presented, the same result was obtained with a completely opposite approach, i.e. selectively enriching a mixed culture. Such approach could be used to enrich different types of inocula under different physico-chemical conditions, with the possibility of discovering other unknown genera able to carry out high rate hydrogen oxidation.

Although not reported within the experimental results, later testing suggested the possible presence of quorum sensing phenomena. Indeed, stored cell used to start up a subsequent reactor system, did not succeed to do so even if their concentration in the biological system exceeded more than 1 g CDW/L. Actually, only when such stored cells were inoculated at about 2 to 3 g CDW/L, the biological process could be restarted. This could of course relate decay and aging, but also to the need to be engaged with numerous partner bacteria in the process of the hydrogen oxidative “convivium”. The role of such community triggering aspects is known to be relevant amongst other very specialized bacterial consortia such as anammox bacteria [327], and it might be speculated that also the intensive expression and production of delicate hydrogenase enzymes, by the enriched microbial community is regulated by a strong cell-to-cell coordination. Further investigation should shed light on such important aspect. Certainly, the aspect warrants more in depth analysis in the future because it is of crucial importance for the in-practice propagation of the HOB-technology.

8.1.2 Future work

On the basis of the findings outlined from this work, additional research should be carried out, aiming at investigating the collaboration within the core of the highly

enriched microbiome, i.e. the HOB and the heterotrophic satellite organisms. Innovative approaches in synthetic microbiology [328] could allow testing and improving the collaborative capacities within the microbiome, assembling specific microbiomes specialized to carry out specific functions at higher rates, or also being more resistant to external invasion. An example of possible core-satellite interaction which could be used as model to guide synthetic community engineering is presented in Figure 8.1. Accordingly to such approach, the synthetic community could be engineering by using satellites able to support and enhance the functionality of the core. At the same time, the satellites could grow by benefitting from an exchange of metabolites or other factors with the core, therefore maximizing the productivity of the biological system.

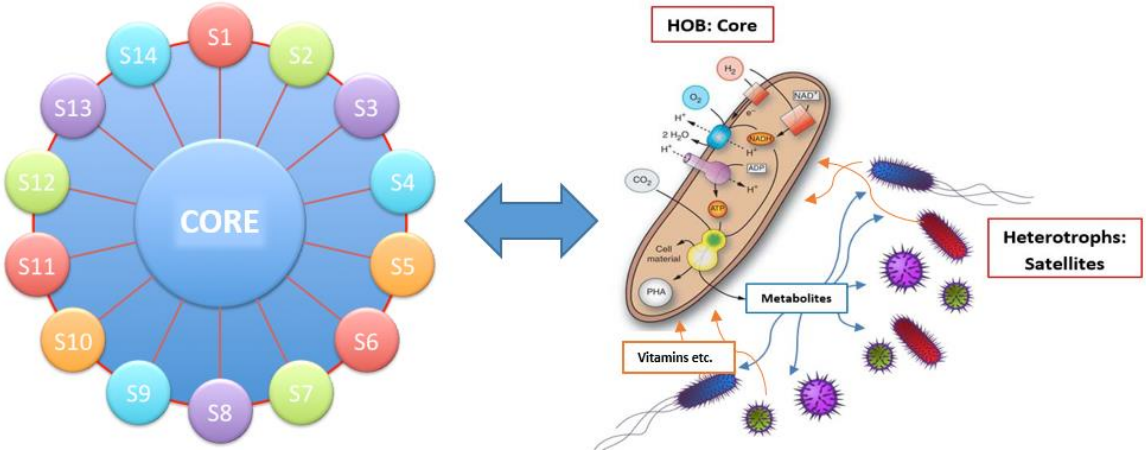

Figure 8.1. *Core–satellites model proposed to explain the possible collaboration within the microbiome. The same model would be adopted to construct possible synthetic microbial communities, assembling different cores with different satellites.*

An important aspect which will need to be covered in the future for the MP platform in general and for the HOB microbiome in particular, is the feed and food safety aspect. An established scientific literature exists and extensive research efforts have been made in the direction of identifying the risks associated to toxins in microbial protein. Risks associated to feed and food-stuff produced by fungi capable of expressing mycotoxins and peptides have been intensively studied [6]. Mycotoxins in feed and food are known to cause a series of disorders, spanning from allergic reactions to neurotoxicity. Their removal has been shown to be possible, especially regarding aflatoxins. Bacterial toxins can be present as endo- or exotoxins, and were found to be

fatal for laboratory animals at nanogram levels, and result much more difficult to remove once present in the produced MP [6]. For the future application of MP produced from the HOB microbiome it is therefore of crucial importance to screen the whole range of bacteria for the putative production of endo- or exotoxins. The presence in substantial amounts of any toxin expressing bacterium must be avoided, and in this sense the engineering of synthetic communities employing non-toxic bacteria is perspective for the application of such platform in feed and food. At the level of microbial community and its evolution it will also be important to assess the risk associated to horizontal gene transfer from any possible toxic external microorganisms. Dealing with a mixed bacterial community for the production of feed and food additives will require that any external invasion will have to be constantly controlled and avoided. Even the temporary presence of any external invader, possibly capable of transferring genes for the expression of toxins to bacteria previously free from such feature must be avoided. In this sense, the selected or further synthetically engineered community must be able to resist to external invasion and avoid any direct or indirect contamination also in terms of horizontal gene transfer. Finally, the safety of the product must be also guaranteed against any possible external chemical contamination; this is indeed a relevant aspect when dealing with the use of recovered resources. The absence of any harmful contaminant in those sources must be constantly controlled in order to guarantee the safety of the final product. All of this is part of what is called “Good Manufacturing Practice” (GMP).

From a technological point of view, the main questions not tackled in the present work involve the downstream processing of the produced microbial biomass, which requires dedicated research and further investigation. Currently, several techniques are implemented for the downstream processing of microbial protein, involving different steps such as dewatering, biomass inactivation by means of heating, drying and palletization.

The dewatering step is amongst the most crucial for the overall feasibility of the process. Dewatering is commonly carried out by means of centrifuging devices. The biomass concentration during the bioconversion represents already an important variable, impacting on the efficiency of the dewatering step. Microbial fermentation processes achieving 2-3% cell dry weight during the cultivation step, allow a more efficient dewatering process, with the final product achieving up to 10-20% dry weight.

In case the biomass concentration in the fermentation broth would be lower, the addition of coagulant and flocculants agents is normally required prior to the dewatering. Nevertheless, such addition can hamper the application of the final product as feed additive due to the presence of non-feed grade material.

The heat treatment is mainly foreseen for biomass inactivation and further for decrease of the nucleic acid content. While the first is strictly necessary and is carried out at higher temperature (120-140 °C) for a few minutes, the second is not always required. Lowering the content is necessary in case the microbial biomass is used for human nutrition. In fact the high levels of nucleic acid would result in issues such as kidney stones and gout [6].

The drying process represents a crucial step in the preparation of the final product. Aiming at a final dry matter content of about 85% upwards, the amount of energy which must be invested for drying depends on the amount of water which was eliminated during the dewatering step. Actually the rule of thumb for drying dewatered microbial biomass is that about 0.85 kWh thermal energy is required per kg water removed. Clearly a dewatered biomass at 20% dry matter needs some 80-90 kWh thermal energy per 100 kg initial wet material. The latter results in 80-90 kWh thermal energy at some 0.05 €/kWh thermal energy per 20 kg dry matter or some 0.25 €/kg end product.

Overall, it is evident that the output of the bioconversion phase in terms of maximum biomass concentration achieved is crucial for the technical and economic feasibility of the consequent downstream processing steps. At the same time though, the concentration of the fermentation broth cannot be increased above a certain limit, defined by the increase of viscosity and the consequent gas mass transfer limitation imposed to the system above concentrations of about 30 gCDW/L.

8.2 The bird's eye view: upgrading recovered nitrogen to replace EU soy imports

The approach proposed, analysed and discussed all over the present work, foresees the implementation of microbial protein production as alternative to conventional protein sources. Currently, soy represents the main vegetable protein used as feed additive for livestock production, and its increasing production in tropical region of the

world is causing serious concerns in term of deforestation, greenhouse gas emission and eutrophication. With a negligible internal production, Europe is amongst the main global soy importers. Current annual EU soy imports are of the order of 20 MT per year, satisfying about 97% of the overall European protein-rich feed materials requirement [329]. Considering a protein content of 40%, this corresponds to about 8 MT protein, or about 1.3 MT N which end up as feed supplement in the livestock sector. As demonstrated along the present work, upgrading of used nitrogen to microbial protein could offer a valid alternative to conventional protein production. Recent estimates report that in EU about 501 million inhabitants are connected to primary, secondary and tertiary wastewater treatment systems, corresponding to a load of 621 million i.e.. This corresponds to an annual collection and treatment of about 2.9 MT N. Assuming that 65% of the waste incoming N could be recovered, this would mean that if less than 70% of the wastewater treatment plant of Europe would recover and upgrade N into microbial protein, this would be enough to make EU totally independent from soy imports.

If as already calculated in chapter 6, the conversion of biomethane into hydrogen, heat and electricity would allow to upgrade about 11% of the total recovered N, about 1.1 MT N would still need to be upgraded to achieve the goal of 1.3 MT N per year under microbial protein form to be used as soy replacement. To upgrade such recovered N by means of the hydrogen-based platform, about 5.2 MT H_2 would be needed, corresponding to a demand of 211 TWh for its generation by means of water electrolysis. The latter could be provided by dedicated renewable energy installations, having the advantage of being placed directly on-site of the WWTP, or also by making use of the excess energy inherently generated by renewable energy systems supplying energy to the net.

Indeed, increasing penetration of renewable energy into the grid will pose severe issues of temporary energy surplus and elegant solutions to this problem are needed. Simulations over the possible future energetic scenario of Europe suggest that if the auspicated total conversion of fossil fuel-based energy generation to renewable energy systems will happen, strong unbalances between offer and demand will be faced.

***Table 8.2**. Simulated scenario of 100% renewable energy sources (RES) generation in EU. Adapted from Pensini et al.* [330]

		EU @ 100% RES	Units
	Total annual EU average electrical consumption (2007)	3240	TW h_{el}
Hypothetic scenario: *100% renewable energy (70% from wind and 30% from solar energy) with minimized storage potential*	Total annual average renewable generation	4860	TW h_{el}
	Total renewable installed capacity	2130	GW_{el}
	Installed storage capacity	220	GW_{el}
	Excess energy	50	% annual consumption
		1620	TW h_{el}

Table 8.2 resumes a hypothetical future scenario where 100% energy is generated through renewable energy, with the amount of energy generation and the relative installed capacity needed to satisfy EU electrical consumption while minimizing the need for energy storage systems. Indeed, the inherent fluctuation of renewable energy generation represents the biggest hurdle to a complete conversion of the current fossil fuel based system. It has been shown that a more cost effective and technologically feasible alternative to energy storage is the installation of higher capacity of renewables. Therefore, generating more electricity than required appears to be the optimal solution to allow a high penetration of renewable energy into the market [330]. In the example here discussed, seasonal variation of renewable energy generation has been balanced by considering a system based for 70% on wind and for the remaining 30% on photovoltaic energy. Nevertheless, even when an optimal balance between energy sources is carefully considered, about 50% of the annual EU energy consumption would need to be produced in order to meet the electricity demand, being such excess energy fated to be wasted. Such a high percentage is due to the inherent daily variation of renewable energy generation, depending on variation of whether (wind and solar energy) as well as on day and night (solar energy).

Therefore, the calculated 211 TWh would correspond to about 13% of the overall surplus energy that would be generated in a future scenario where 100% of energy would be provided by means of renewable sources such as wind and solar energy.

In such context, the challenges posed by the efficient management and integration of renewable energy will might also offer unforeseen opportunities in upgrading recovered nitrogen.

8.3 The pragmatic point of view

8.3.1 Political and societal barriers

Recovery of nitrogen and upgrade into microbial protein is a very compelling concept when it comes to the circular economy. The latter constitutes a politic issue which is currently in the spotlight, and is intended to become more and more important in the near future. Policies promoting sustainable technologies that allow economic growth and job creation are also more and more frequently adopted. Furthermore, the perspective of generating high-quality protein on-site offers the major advantage of becoming independent from imports and global market fluctuations. Overall, future political decision seem to be propitious for the establishment and integration of microbial protein production from recovered resources in the years to come.

From a societal point of view, the production of microbial protein has the added benefit of potentially engaging the public in a positive sense. If the broader public can perceive a benefit in turning waste into a valuable resource that preserve or increases the quality of life while at the same time aiding the environment, it can more likely become receptive of the technology. Placing the production of microbial protein as a viable technological and sustainable solution to conventional resource intensive protein production, can attract public interest and promote the widespread adoption of such technology. The adoption of incisive dissemination strategies engaging local actors and potential end-users, as well as the implementation of policy relevant assessments such as the IPCC reports can furthermore help achieving a widespread public acceptance towards microbial protein from recovered resources. High importance must be given to highlighting how such alternative protein source can actually be categorized within already established nutritional habits such as the consumption of fermented foodstuff like cheese, beer, yoghourt, etc.

Overall, society has therefore a positive attitude towards technologies that support environmentally friendly processes. However, attention will have to be paid to provide sufficient information regarding the quality of the treated wastewater, the safety and social acceptance of the obtained product as well as the overall sustainability of the process. Special attention needs to be given to providing detailed and accurate information on the quality and control strategies in place to assure that a high-quality product is achieved at all times. Finally and foremost, very detailed attention will have

to be given to cultural attitudes. Indeed, when the ammonium recovered from wastewater, will be upgraded to feed, objections could be raised by certain cultural-religious groups and these concerns will have to be respectfully addressed in a proper way. The best strategy to be adopted in this case should consist in providing open and transparent communication and gradually gaining confidence in the context of quality assurance.

8.3.2 Legislative barriers

Microbial protein can be already brought to the market as animal feed, thanks to an already existing legal framework. Of course, legal requirements for the introduction of novel foods/feeds on the market require obligatory testing which must be performed prior to final consideration of the microbial protein as feedstuff, including allergens, nutritional value and digestibility. Relevant in this context are the Novel Food regulation (EG) nr. 258/97 and for the admission of new products as animal feed the part 6 of Regulation 767/2009/EG, which includes the exploration of regulatory issues which could be encountered on the way to market application in different countries. Of great importance is the implementation of compulsory risk assessment studies, mainly focussing on the toxicity of novel feed and food. The risk associated to MP must be well established towards any possible allergenic toxicological endpoint such as for example allergenic reactions or immune effects. Several studies have already been carried out on the toxic effects of MP products on different types of animals and humans as well [6], some of them resulting in adverse health effects directly linked to the specific MP product use. This studies, and the aspects highlighted must be carefully taken into account while dealing with the establishment of novel feed or food products such as MP. Feed trials and animal studies are therefore a necessary milestone towards the achievement of novel feed or food status.

Overall; in order to enter the feed market microbial protein must be produced in a way conform to already established procedures used to produce micro-organisms fit for feed purposes (for instance feed fodder yeast). The products must be manufactured in accordance to good manufacturing practices (GMP) and verified for feed quality prior to use in animal tests.

The following steps can be foreseen to ensure the appropriate conditions for the production and legal approval of microbial protein:

1. Confirmation of the competing legal authority (In case of EU, EFSA: European Food Safety Authority)
2. Introduce GMP in the production process (production facilities, personnel, procedures and documentation)
3. Acquire the GMP certification
4. Quality control and quality assurance

8.3.3 Economic considerations

The economic considerations presented in this work are meant to offer only a preliminary overview about possible final cost of the MP product and its comparison with other conventional protein sources. Different factors should be more carefully taken into account in order to deliver a more clear and robust comparison.
From the point of view of already established and marketed products such as e.g. soybean or fishmeal, prices vary greatly when different markets are considered as well as if direct or indirect subsidies are applied or not.
Those factors do not apply to novel products resulting from alternative technologies. The latter are affected by another range of variables and uncertainties, such as learning effects, efficiency gains and scale of economies, aspects which might drive the future price of alternative new products far from what it is foreseeable at such early stage.
Based on the technology outlined in the present work for the production of MP by HOB, it is already possible to enumerate some of the main variables which play a key role in the definition of the future price of the MP.
Considering the energetic substrate for MP production, the cost of hydrogen from water electrolysis will be greatly affected by the fluctuation of future electricity prices, the efficiency gain of industrial electrolysis modules, and any possible subsidy to renewable hydrogen generation. Fermentation technology is also a crucial aspect. The implementation of innovative and more efficient techniques for gas transfer will allow to decrease aerobic fermentation costs, as already demonstrated e.g. by the innovative reactor concept developed by Unibio A/S [147].
As already outlined in paragraph 8.1.2, downstream processing represents a very important process step, affecting the technical but also the economic feasibility of the

final product. Steps such as dewatering and drying can sensibly impact the final production cost, especially depending on the electricity price and on the eventual availability of local cheap energy sources such as for example otherwise wasted low value heat.

Finally, the establishment of a circular economy considering MP production from recovered resources as a more sustainable alternative to conventional protein sources, might lead to the creation of direct or indirect subsidies which could contribute further to the definition of the final market price of the MP product.

A sensitivity analysis taking these aspects into account would allow to evaluate more critically the assumptions which were made in this study, and would serve as base for further development of more thorough economic evaluation on the MP platform and its application in the various contexts outlined in the present work.

8.4 Future challenges and perspectives

Although the rate of population growth is estimated to decrease, the overall world population is still going to rise and numbers in the 9-10 billion range have to be expected. A higher fraction of the population will require access to highly nutritious protein. The clean-tech microbial protein can help to alleviate these needs, either indirectly by allowing producing more animal protein, over even directly as a form of food such as at present already known in the form of microbial ferments such as yoghurts, yeasts, and mushrooms .Clearly, a crucial issue to be addressed is informing the public correctly about the nature and the nutritional value of these -already existing and well documented - sources of microbial protein and particularly about the benefits which their more extensive production constitutes for both the consumer (new supply route at competitive costs) and for the environmental sustainability (much lower externalized costs) . Particularly for the direct supply route of microbial protein to human food, an additional challenge will be to create product formulations which have a competitive texture and taste, as for instance in the case of Quorn™.

A second element is that the current carrying capacity of the planet in terms of producing more plant based protein is reaching its limits. Although still more ha of land (also marginal land) can be brought to intensive production of protein rich crops; the overall burden on the environmental balance will be very debatable. Moreover, the

microbial protein approach alleviates the need for GMO removing this issue from the public debate.

The third factor is, as referred before, the absolute need to address the third in rank boundary condition of our planet. The production of microbial protein directly derived from our approach, as schematized in Figure 8.2, deviates the mass flow of reactive nitrogen input in the biosphere to the reactor based protein production at unmatched efficiencies of nitrogen to protein of near 100%.

Finally, there are at present major technological breakthroughs going on in the domains of green energy, hydrogen production and biotechnological conversion of hydrogen to high value microbial cellular components such as amino acids, alkanoates, single cell oils, and exo-cellular polymers. These new developments will empower the processes with various insights and improvements to make them fit even more coherently within the framework of this alternative clean-tech protein supply chain.

8.5 Concluding remarks

The supply of quality protein is of crucial importance for the overall health and wellbeing of the populations worldwide. The conventional routes to produce protein rich feeds and foods give rise to substantial environmental pressures, especially in terms of reactive nitrogen flows. To alleviate this protein-environmental nitrogen nexus, an alternative platform should make use of Haber Bosch nitrogen remains as the key driver, but also focusing on the recovery and subsequent upgrade of used nitrogen from industrial and domestic side streams (see Figure 8.2).

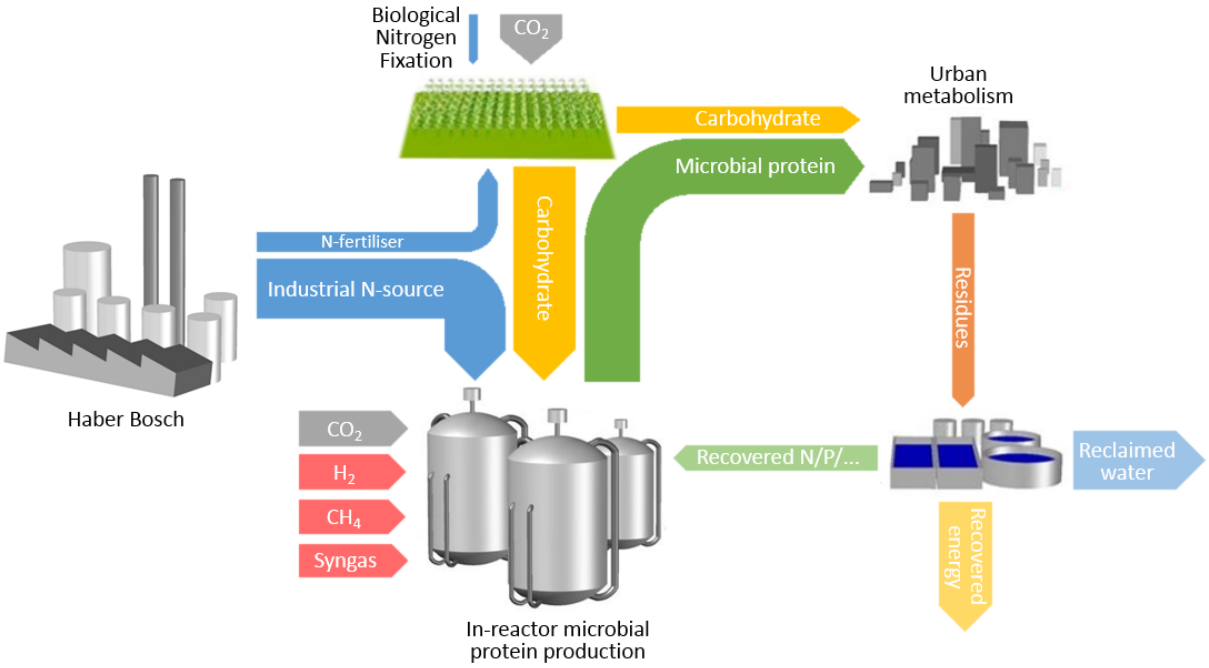

***Figure 8.2**. Low Haber Bosch N inputs for crops and both Haber Bosch and used nitrogen for microbial protein production to make feed and food*

The proposed platform particularly integrates established knowledge in relation to the concept of microbial protein with new insights in green energy, biotechnology, hydrogen generation from green energy and process technology.

The main advantages of such platform is the complete independence from issues affecting the conventional feed and food chain such as GMOs, special chemicals and unaffordable water uses or and land footprints. It also does not create tensions in terms of north/south agro productions since it can be driven each time by the resources present and producible in loco (carbohydrates, renewable energy, natural gas etc.). Cleary, this new platform holds considerable challenges in terms of the biotechnological issues of growing, harvesting, quality assurance of the final microbial products, especially when destined for human consumption, and it particularly will depend on a careful and correct dialogue in terms of overall acceptability with the regulators. With the public at large, the key issue is to emphasize the mutual benefits for the consumer and the environment alike of this new platform.

Abstract

ABSTRACT

The current feed and food supply chain, providing high-quality protein mostly to the western world, has as it stands today trespassed global sustainability boundaries several times. With the human population expected to reach 10^{10} individuals within the next 30 years, and with major developing countries approaching higher standards of life, the demand of high-quality protein is meant to steeply increase, thus increasing current global environmental unbalances.

The search for alternative protein sources, able to amend the inefficiency and the environmental impact of conventional feed and food sources, is currently moving intellectual and physical resources globally and at multiple levels. Fundamental research, biotech industry, environmental scientists and last but not least, governmental and legislative bodies are all actively working towards the establishment of alternative feed and food platforms. Amongst them, microbial protein has been already the object of intense research and development efforts, with existing examples of full scale and market applications both as animal feed but also as human food (Quorn™ products). Such in-reactor based process offers substantial environmental advantages compared to the conventional land-based protein production, especially in terms of land and water footprint, but also in relation to greenhouse gas emission and nutrient management, with the latter being an aspect frequently overlooked. In fact, the direct upgrade of reactive nitrogen, either synthesized from Haber Bosch or recovered from used streams, would offer a serendipitous short-cut to the highly inefficient anthropogenic nitrogen cycle.

The vast array of microbial actuators, metabolic pathways and production methods offers the possibility of using different approaches and combinations. Bacteria, with their metabolic flexibility, high growth rates and protein content are amongst the most promising microbial protein sources.

This study focuses on the production of microbial protein by means of a generic culture selectively enriched in hydrogen oxidizing bacteria. This specific group of bacteria was chosen because of their capability to oxidize molecular hydrogen while fixing carbon dioxide and upgrading mineral nitrogen into high-quality microbial protein. Moreover,

the use of a mixed culture instead of axenic cultures allowed exploring innovative microbial production methods, free from the strict sterility measures currently employed in conventional biotech industry.

The work is structured at different levels, starting from the most fundamental aspects of the enrichment procedure and its outcomes, continuing with the biotech performances of the enriched microbiome and being completed with more general evaluations of this as well as other microbial protein platforms in the context of resource recovery and global environmental sustainability.

Chapter 2 introduces hydrogen oxidizing bacteria from their fundamental physiological characteristics, also outlining their biotech potential. Recovered nutrients as well as hydrogen and oxygen, produced by green or off-peak energy by electrolysis are proposed as raw material for the sustainable production of high value bio-products such as microbial protein and biopolymers. Based on literature data such as biomass yield, hydrogen prices and operational expenses, a preliminary estimation on the cost of microbial protein by means of HOB has been made. The estimated production costs were found to be higher than soybean meal but in line with high quality protein sources such as yeast protein grown molasses. In this chapter, an overview on nutrient recovery techniques highlights how the current costs of nitrogen recovery can hamper the economic feasibility of microbial protein produced by upgrading such used nutrient source.

In **chapter 3** the approach foreseeing the use of microbiomes, instead of axenic cultures, is introduced. First the methodology used to enrich the HOB from an environmental sample is presented and then the results of the enrichment process are discussed. The effect of headspace oxygen levels on the biomass yield as well as on gas consumption was investigated, demonstrating its major influence on the hydrogen oxidation metabolism. Higher headspace oxygen levels clearly led to increased gas utilization, yet the correspondent biomass yields were lower. Under lower oxygen levels, gas utilization was instead lower, but the response in biomass yield was higher. Furthermore, the enriched culture was tested under different physico-chemical conditions in microtiter plate experiments, confirming some kinetic features typical of HOB. Finally, molecular analysis on the enriched microbial community revealed a strong balance between hydrogen oxidizing bacteria (HOB) and other bacteria within the established microbial consortium.

Chapter 4 presents and discusses the bench scale experiments conducted on the enriched HOB microbiome. The main biotech parameters such as biomass yield, volumetric productivities and gas utilization efficiency are compared between two specific engineered reactor systems: the sequencing batch reactor (SBR) and the continuous reactor (CR). At low selection pressure (i.e. under sequencing batch culture at high solid retention time), a very diverse microbiome with an important presence of putative parasite *Bdellovibrio* spp. was observed. The microbial culture which evolved under high rate selection pressure (i.e. dilution rate $D=0.1h^{-1}$) under continuous reactor conditions was dominated by *Sulfuricurvum* spp. and exhibited a highly stable and efficient process in terms of N and C uptake, biomass yield and volumetric productivity. Under continuous culture conditions the maximum yield obtained was 0.29 g cell dry weight per gram chemical oxygen demand equivalent of hydrogen, whereas the maximum volumetric production rate peaked 0.41 g cell dry weight per litre per hour at a protein content of 71%. The microbial protein produced was of high nutritive quality in terms of essential amino acids content, thus representing a suitable substitute for conventional feed sources such as fishmeal or soybean meal.

In **chapter 5** the upcycling of nitrogen recovered from used water for the production of microbial protein by means of HOB and other microbial platforms is discussed. The poor nitrogen use efficiency of the current feed and food chain results in only 17% of the initial agricultural nitrogen input retained in vegetable and meat protein, and 15% in urban wastewater. Nitrogen recovery is discussed and compared with conventional removal technologies, which dissipate reactive nitrogen into the atmosphere employing the same amount of primary energy used by the Haber Bosch process to produce nitrogen fertilizers. In terms of primary energy, the production of microbial protein was estimated to be about one order of magnitude less demanding than the conventional agro-based food production. Under these circumstances, the upcycling of recovered nitrogen as microbial protein would offer important advantages in terms of environmental sustainability and resource efficiency.

Chapter 6 presents a case study where the HOB-based microbial protein platform is implemented in the upcycling of nitrogen recovered from anaerobic digestion. In this case, the “water factory” maximizing C and N recovery is sketched and implemented with renewable hydrogen generation platforms such as the combined heat, hydrogen and power generation (CHHP), used to capture upcycle CO_2 and N under the form of

microbial protein. By exploiting the intrinsic energy content of the used water, the process scheme here depicted would be capable of up-cycling up to 11% of the total nitrogen recovered from the treatment plant directly to valuable microbial biomass rich in edible protein.

In **chapter 7**, different microbial platforms for the production of microbial protein are compared. In this case, the production of microbial protein is analyzed starting from industrially synthesized nitrogen, i.e. Haber-Bosch produced nitrogen, allowing a more direct comparison with the conventional agro-feed-food based system. A thorough economic analysis was used as input for the global agricultural model MAgPIE, allowing to confront the different platforms in terms of nitrogen use efficiency, greenhouse gas emissions and land use efficiency on a global scale. The analysis revealed that by 2050, microbial protein could replace around 13% (11–18%) of conventional feed protein demand. Of the different production scenarios considered, the agriculture-free and climate independent production of microbial protein (landless microbial protein production) appeared to be optimal since it allowed to decrease global cropland area expansion by 13% (144 Mha), global nitrogen losses by 9% (12 Mton N_r) and greenhouse gas emissions by 8% (46 Gt CO_2 equivalents).

The microbial protein platforms overall considered and discussed in this work, and the hydrogen oxidizing microbiome object of scientific research and development, hold considerable potentials in guaranteeing a more sustainable supply of quality protein for the health and wellbeing of the populations worldwide.

Multiple challenges still await. Future work should address microbiological and biotechnological issues, such as stability of production under non-axenic environments, but also technical aspects such as harvesting and quality assurance of the final microbial products.

The final establishment of microbial protein production will also depend on a careful and correct dialogue in terms of overall acceptability with the regulators and with the public at large.

SAMENVATTING

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De huidige voeder- en voedselketen, die voornamelijk het Westen van hoogwaardig eiwit voorziet, overschrijdt heden ten dagen verscheidene malen de draagkracht van de wereld. Gezien verwacht wordt dat de globale populatie zal oplopen tot 10 miljard mensen binnen de 30 jaar, en belagrijke ontwikkelingslanden een steeds hogere levensstandaard bereiken, zal de vraag naar hoogwaardig eiwit sterk toenemen waardoor de huidige ecologisch onbalans nog verder uit evenwicht zal worden gebracht.

De zoektocht naar alternatieve eiwitbronnen, die een antwoord moet bieden op de huidige inefficiënte en milieu-impact van de conventionele voeder en voedselbronnen, brengt momenteel een beweging teweeg, zowel op intellectueel als op fysisch vlak en dit op verschillende niveaus. Fundamenteel onderzoek, biotech industrie, milieudeskundigen en overheids- en wetgevende instanties werken allemaal actief aan het opbouwen van alternatieve voeder en voedsel platformen. Microbieel eiwit werd reeds intens onderzocht en ontwikkelend, met voorbeelden van volle schaal productie en marktapplicatie als resultaat, niet enkel als bij voorbeeld voeder, maar ook als voedsel (Quorn^{TM} producten). Dergelijke reactor gebaseerde processen bieden verschillende specifieke voordelen in vergelijking met land gebonden eiwitproductie, voornamelijk op het gebied van land- en watergerbruik maar ook broeikasgas-uitstoot en nutriëntenbeheer – waarbij dit laatste euvel vaak vergeten wordt. Inderdaad, het directe opwerken van reactieve stikstof, gesynthetiseerd via Haber Bosch of gerecupereerd uit reststromen, zou een kans kunnen bieden om de erg inefficiënte antropogene stikstofcyclus drastisch te verbeteren.

Het brede gamma aan microbiële actoren, metabolische routes en productiemethodes bieden de mogelijkheid om verschillende benaderingen en combinaties te gebruiken. Bacteriën, met hun metabolische flexibiliteit, hoge groeisnelheden en aantrekkelijk eiwitgehalte, behoren tot de meest veelbelovende microbiële eiwitbronnen.

Deze studie richt zich op de productie van microbieel eiwit door middel van een generische cultuur die selectief is aangerijkt met waterstof oxiderende bacteriën. Deze specifieke groep van bacteriën werd gekozen omwille van haar capaciteit om moleculaire waterstof te oxideren teneinde CO_2 te fixeren en minerale stikstof op te

waarderen tot hoogwaardig microbieel eiwit. Bovendien staat het gebruik van een gemengde microbiële cultuur in plaats van een axenische cultuur toe om innovatieve en kosten/baten gunstige microbiële productiemethoden te verkennen.

Het werk werd gestructureerd op verschillende niveaus, startende meet de fundamentele aspecten van de aanrrijkingsprocedure. Aansluitend wordt de biotechnologische prestatie van het aangereikte microbioom onderzocht. Tenslotte worden ook andere microbiële eiwitplatformen in de context van grondstofrecuperatie en globale duurzaamheid geëvaluerd.

Hoofdstuk 2 introduceert waterstof oxiderende bacteriën en schetst hun fundamentele fysiologische kenmerken, alsmede hun biotech potentieel. Herwonnen voedingsstoffen, evenals waterstof en zuurstof, geproduceert door middel van elektrolyse op basis van off-peak groene energie worden voorgesteld als grondstof voor de duurzame productie van hoogwaardige bio-producten zoals microbieel eiwit en geassocieerde biopolymeren. Op basis van gegevens uit de literatuur zoals biomassa opbrengst, waterstof prijzen en operationele kosten, is een voorlopige schatting van de kosten van microbieel eiwit door middel van HOB uitgewerkt. De geraamde productiekosten bleven hoger dan deze van sojameel, maar in lijn met hogere kwaliteit eiwitbronnen zoals gist eiwit gegroeid op basis van melasse. Een overzicht van de technieken om voedingsstoffen te herwinnen uit reststromen geeft aan dat de huidige kosten van ammonia captatie de economische haalbaarheid van microbieel eiwit geproduceerd kan beperken.

In **hoofdstuk 3** worden axenische culturen als insteek voor opbouw van microbiomen geïntroduceerd. Eerst worden de HOB vanuit milieu stalen aangerijkt en vervolgens worden de resultaten van het verrijkingsproces besproken. Het effect van het zuurstofgehalte in het kopgas van de reactor op de biomassa opbrengst en op gasverbruik bleek belangrijk te zijn. Hogere zuurstof geeft een verhoogd gebruik van gas, maar de opbrengsten aan biomassa zijn lager. Bij lagere zuurstofgehalten is gas verbruik lager, maar de opbrengst van biomassa is hoger. Bovendien werd de aangerijkte kweek getest onder verschillende fysisch-chemische omstandigheden. Microtiterplaat experimenten, bevestigden bepaalde kinetische eigenschappen typisch voor HOB. Tevens werd een eerste microbiologische analyse van het verrijkte microbiële team doorgevoerd. Er blek een sterk evenwicht tussen waterstof

oxiderende bacteriën (HOB) en andere bacteriën binnen het vastgestelde microbieel consortium.

Hoofdstuk 4 presenteert en bespreekt de grootschalige experimenten uitgevoerd met het verrijkte HOB microbioom. De belangrijkste biotech parameters zoals biomassa opbrengst, volumetrische productiviteiten en efficiëntie van gasverbruik worden vergeleken voor twee specifieke engineered reactor systemen: een stelsel van opeenvolgende batch reactoren (SBR) en de continue reactor (CR). Bij lage selectiedruk (dat wil zeggen bij een hoge biomassa retentie tijd), werd een zeer divers microbioom met een belangrijke aanwezigheid van potentieel parasitaire *Bdellovibrio* spp. waargenomen. De microbiële cultuur die zich ontwikkelde onder hoge selectie druk (dat wil zeggen verdunning D = 0,1 h^{-1}) onder continue reactor omstandigheden werd gedomineerd door *Sulfuricurvum* spp.. Aldus werd een zeer stabiel en efficiënt proces bekomen in termen van N en C-opname, de opbrengst aan biomassa en volumetrische productiviteit. Onder continue kweekomstandigheden was de maximale verkregen opbrengst 0,29 g cel drooggewicht per gram chemisch zuurstofverbruik equivalent van waterstof, terwijl de maximale volumetrische productiesnelheid piekte aan 0,41 g cel droog gewicht per liter per uur bij een eiwitgehalte van 71%. Het microbiële eiwit had een hoge voedingswaarde kwaliteit in termen van essentiële aminozuren, zodat het als vervanger voor conventionele voedingsbronnen zoals vismeel of sojameel in aanmerking komt.

In **hoofdstuk 5** wordt de upcycling van stikstof gewonnen uit proces water voor de productie van microbieel eiwit door middel van HOB en andere microbiële platforms besproken. De lage stikstof efficiëntie van de huidige voedselketen heeft voor gevolg dat slechts 17% van de oorspronkelijke ingebrachte agrarische stikstof in voedingseiwit terecht komt en 15% finaal eindigt in stedelijk afvalwater. Hergebruik van ammonium stikstof werd met het aanmaken van Haber Bosch proces stikstof vergeleken op basis van de gangbare technologieën. In termen van primaire energie werd de productie van microbieel eiwit berekend ongeveer een orde van grootte minder veeleisend te zijn dan conventionele agro gebaseerde voedseleiwit productie. Onder deze omstandigheden kan de upcycling van teruggewonnen stikstof als microbieel eiwit belangrijke voordelen bieden op het gebied van ecologische duurzaamheid en efficiënt gebruik van hulpbronnen.

Hoofdstuk 6 presenteert een case studie waarin het HOB-gebaseerde microbieel eiwit platform is toegespitst op de upcycling van stikstof, herwonnen via anaerobe

vergisting. In dit geval wordt de "water factory" gericht op de maximale C en N terugwinning en worden hernieuwbare waterstof platforms zoals warmte, waterstof en energieopwekking (CHHP) gebruikt om upcycling van CO_2 en N door te voeren in de vorm van microbieel eiwit . Door het benutten van de intrinsieke energie-inhoud van het gebruikte water, kan het vooropgestelde processchema instaan om 11% van de totale stikstof direct gewonnen uit de zuiveringsinstallatie om te zetten tot waardevolle microbiële biomassa rijk aan eetbaar eiwit.

In **hoofdstuk 7** worden verschillende microbiële platformen voor de productie van microbieel eiwit vergeleken. In dit geval wordt de productie van microbieel eiwit geanalyseerd vanuit industrieel aangemaakte reactive stikstof, d.w.z. Haber-Bosch gevormde stikstof. Er wordt een directe vergelijking doorgevoerel met het conventionele agro-feed-food systeem. Een grondige economische analyse wordt gebruikt als input voor het wereldwijde landbouwmodel MAgPIE. Dit model maak het mogelijk om de verschillende platformen te confronteren in termen van stikstof efficiëntie, de uitstoot van broeikasgassen en landgebruik efficiëntie op een wereldwijde schaal. Uit de analyse bleek dat in 2050, microbieel eiwit ongeveer 13% (11-18%) van de conventionele voeding eiwit vraag kan vervangen. Van de verschillende productie-scenario's die werden onderzocht bleek de landbouw-vrije en klimaat onafhankelijke productie van microbieel eiwit (landloze microbieel eiwit productie) optimaal. Deze kan resulteren in de grootste daling in de wereldwijde akkerland gebied expansie door 13% (144 Mha), de wereldwijde stikstofverliezen door 9% (12 Mton Nr) en de uitstoot van broeikasgassen door 8% (46 Gt CO2-equivalenten).

De microbieel eiwit platformen algemeen beschouwd en besproken in dit werk, samen met het waterstof-oxiderende microbioom als voorwerp van wetenschappelijk onderzoek en ontwikkeling, houden aanzienlijke mogelijkheden voor het waarborgen van een duurzame samenleving voorzien van hoogwaardige eiwitten voor de algehele gezondheid en het welzijn van de bevolking.

Meerdere uitdagingen moeten worden geadresserd en wachten op verdere investigatie. Op het gebied van microbiologische en biotechnologische kwesties, is de vraag van de stabiliteit van de productie onder niet-axenic omstandigheden heel balangerijk. Op technisch vlak is het oogsten en de kwaliteitsborging van de uiteindelijke microbiële producten een punt van zong.

De definitieve doorbraak van microbiële eiwitproductie zal ook afhangen van een zorgvuldige en correcte dialoog in termen van algemene aanvaardbaarheid met de wetgevers en toezichthouders en met het grote publiek.

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CURRICULUM VITAE

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Silvio Matassa holds a master in Environmental Engineering obtained in 2013 at the University of Cassino, Italy. From January 2014 till the current date he was an industrial PhD researcher at Avecom NV, Belgium. In the same period, a close scientific collaboration was established and maintained with the Center of Microbial Ecology and Technology (CMET), Ghent University. During his PhD he has investigated the microbiological aspects as well as the biotech potentialities of hydrogen oxidizing bacterial microbiomes for protein production. Both fundamental research in the domain of microbial and environmental biotechnology as well as research and development within an industrial environment have guided his work. During his PhD he has guided and supported 4 internship and 1 thesis student. As part of a multidisciplinary European scientific training network, he was involved in several scientific and non-scientific training activities across Europe. Also, as result of the research and development industrial activity, he was directly involved in the development of a multinational project regarding the upscaling of hydrogen oxidizing bacteria-based microbial protein production from recovered nitrogen.

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SCIENTIFIC CONTRIBUTIONS

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